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The Effects of Petroleum Pollutants on Sea Urchins Reproduction and Development

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THE EFFECTS OF PETROLEUM POLLUTANTS ON SEA URCHINS REPRODUCTION AND DEVELOPMENT

By

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Abstract

Disturbances, such as mass pollution events, threaten the health of vulnerable ecosystems. Recent media attention has focused on the devastating mass oil spills, but daily petroleum input from recreational and commercial ship bilge release has been overlooked. The focus of this study was the effect of petroleum products found in bilge water on fertilization success and larval viability of two sea urchin species, *Lytechinus variegatus* and *Eucidaris tribuloides.* Unlike other pollutant studies that have focused on sperm characteristics and concentrations, I chose to examine how egg integrity was compromised by petroleum products. Scanning electron microscopy revealed eggs were degraded when exposed to low levels of these pollutants. Of the three, oil was the most detrimental to *Lytechinus variegatus* fertilization, while gas was highly detrimental to *Eucidaris tribuloides*. Dosing the eggs for only two hours before introducing sperm demonstrated significant reduction in fertilization and larval survivorship. These data suggest that even relatively low and brief exposure to petroleum pollutants can have devastating effects on sea urchin reproductive success. New regulations may need to be considered when determining the safe petroleum concentration in bilge discharge.

Keywords: Echinoids, Toxicity, Fertilization, Petroleum, SEM

The influences of petroleum pollutants on the reproductive success of two sea urchins species
Chapter 1

1.1 The ecological effects of oil spills

The ecological significance of oil spills are complex and can have long lasting and persistent effects (Woodley *et al.* 1978). Lethal toxic effects for adult marine organisms when exposed to high concentrations of soluble hydrocarbons range from 1-100 ppm, while the larval stages are significantly more sensitive with a range beginning as low as 0.01 ppm (Moore & Dwyer 1974). Oil spills often contaminate the sediments and can impact the benthic invertebrates for decades (Teal & Howarth 1984).

In recent decades, millions of gallons of oil have been accidently spilled worldwide. To give a few examples, Ixtoc 1 Oil Well in the Bay of Campeche, Mexico spilled 450,000 to 1.40 million gallons of oil in 1979. The same year, the SS Atlantic Express, a Greek oil tanker, collided with another oil tanker and spilled 88.3 million gallons near Trinidad and Tobago. The Exxon Valdez oil spill occurred on March 24, 1989 when a tanker ran aground on the Bligh Reef in the Prince William Sound, Alaska, spilling approximately 11 million gallons of crude oil (Tietenberg and Lewis 2009). In 1991, during the Gulf War in Kuwait, 240-336 million gallons entered the Persian Gulf. Most recently, the Deepwater Horizon rig explosion released 210 million gallons of oil into the Gulf of Mexico on April 20, 2010. Subsequently, 1.84 million gallons of dispersants were applied to dissipate the oil (Cleveland 2010, Dept. of Interior 2012). This was the largest marine oil spill in history. Only 33 million gallons were collected in cleanup efforts (Dorsett 2010). The oil gushed from approximately 5,000 feet below the surface creating underwater plumes of oil throughout the water column and rose to create oil slicks on the surface (Soysa *et al.* 2012).

Despite the environmental damages that oil spills may cause, the long term harm to marine organisms is unclear. After the Exxon oil spill occurred, it was assumed that the oil would dissipate over time and the affected ecosystems would recover. This unfortunately was not the case. Twelve years after the spill, Jeffrey Short, a chemist with NOAA, sampled 91 beaches in the vicinity of Prince William Sound and an estimated

15,000 gallons remained in the intertidal zones mostly below the rocky surface (Short *et al.* 2004). Twenty years later, the new estimate of remaining oil is 16,000 gallons, even more than Short's prediction (Bernton 2009). The rate of dissipation was rapid during the first 3.5 years after the spill, but the rate of dissipation has slowed drastically since then (Short *et al.* 2004). The residual oil has impacted the salmon and the herring fisheries the greatest (Ott 2009). While the salmon population has recovered enough to support the fishery, the herring population is only 15% of what it was prior to the spill and cannot support a fishery (Guterman and Pasotti 2009). Twenty-five years after the spill, clams, mussels, and salmon have recovered, but other organisms like the killer whale pod ATI show few signs recovery. Pod ATI showed a reduction in the reproductive capacity through the loss of mature females and subsequent losses of young offspring (Matkin 2013). These data suggest that the recovery of fragile ecosystems exposed to oil may take decades to recover.

Figure 1.1 Bilge water

Many resources have been allocated to studying the recent Deepwater Horizon spill. Goodbody-Gringley *et al.* (2013) examined the toxicity of the source oil and chemical dispersant, Corexit 9500, on coral larvae. Short and long term exposure to medium and high concentrations of CEWAF [water accommodated fraction (WAF), plus Corexit 9500] resulted in near complete mortality; highly detrimental to settlement and survival of *Porites astreoides* and *Montastraea faveolata* larvae. Soysa *et al.* (2012) found that crude oil severely impaired embryological development (including cardiovascular and craniofacial development) and the locomotion escape in zebrafishes larvae, *Danio rerio*. In 2013, reports of 900 bottlenose dolphins and 500 sea turtles have been found dead or stranded and decreased numbers of tuna, red snapper, and mahi-mahi have been reported in the affected area (NOAA 2014). Invertebrates, like eastern oysters, have experienced particularly high

mortality and low spat recruitment since the spill (NOAA 2013). Five years after the Deepwater Horizon spill, researchers continue to assess the damages to the marine environment.

While oil spills like the Deepwater Horizon and the Exxon Valdez receive high priority because of their national exposure and magnitude, anthropogenic pollutants introduced daily are not widely known (McCook 1999, Nystrom *et al.*., 2000, Bellwood *et al.* 2004). According to Wiggins (2000), close to 3.5 million tons of oil are released into the ocean every year and only a very small percent is attributed to massive oil spillages. For instance, natural oil seeps occur throughout the depths of the oceans attribute to this amount of oil released. However, since the oil seeps are generally very old and flow at a consistently low rate, organisms that live nearby are able to adapt to the toxicity of the hydrocarbons (NAS 2003). The daily release of oil near coastal communities is the most concerning. Nearly 70% of oil pollution estimated by Wiggins (2000) is due to chronic dumping of petroleum waste, including releases of oily bilge water (Fig. 1.1), municipal and industrial wastes or runoff, and other vessel transportation besides tankers. Since there is no consistency on how much petroleum waste is released at a single point, organisms near these areas do not adapt to the pollutants. Thus, most organisms in the water column have no natural defenses against petroleum pollutants (Kvenvolden 2003).

Pollutant levels where ships berth are regulated under the Federal Water Pollution Control Act Amendments of 1972, otherwise known as the Clean Water Act, governed by the Environmental Protection Agency (EPA) and the US Coast Guard (Wiggins 2000). This Act was introduced to the Senate in October 1971. It was passed through the Senate and Congress and later vetoed by President Nixon on October 17, 1972. This veto was overridden by the Senate and Congress consecutively and became law on October 18, 1972. Two major amendments have been added to this act, the Clean Water Act of 1977 and the Water Quality Act of 1987. There have been no additional amendments since 1987. The amount of recreational boaters has almost tripled in the past two decades, therefore so has the amount of bilge water entering the ocean.

The bilge is the lowest part of a ship, which sits below the waterline where the two sides meet the keel. Depending on a ship's design and function, bilge water is the

mixture of water, oily fluids, lubricants, gasoline, cleaning fluids, and other similar wastes that accumulate in the lowest part of a vessel (US EPA 1999). It comes from a variety of sources including the main and auxiliary engines and other mechanical and operational sources found throughout the machinery spaces (spaces containing propelling machinery, boilers, oil fuel systems, etc.) of a vessel. Any water that does not drain off the side of the deck will drain down to the bilge. The amount of bilge water in a ship is highly variable and dependent on factors including the size of the ship, engine room design, preventive maintenance, and the age of the components (US EPA 1999). Bilge water has to be pumped out of the ship, thus it is one of the main sources of oil in the marine environment. Fuel and lube oil in bilge water are made from the distillation of Fuel Oil #2, which is characterized by light density, moderate volatility, and is moderately toxic (Safe Water 2000). The density of sea water is 1025 kg/m^3. Gasoline has a density of 737 kg/m^3 and floats along the surface of the water column and does not disperse easily (Kuppler *et al.* 2009). Fuel oil with a density of 890 kg/m^3 will float but can be easily dispersed throughout the water column or absorbed in suspended solids and deposited onto the benthos (Safe Water 2000).

The International Maritime Organization (IMO) and the EPA specify that bilge water may be discharged into the sea only if its residual oil content is equal to or less than 15 parts per million (ppm) per gallon of bilge water within 12 nautical miles of the coast. The EPA does not restrict the amount of residual oil if it is released outside of 12 nautical miles from the coast (Wiggins 2000). Port Everglades is located off the coast of Florida

Table 1. Port Everglades Waterborne Commerce Chart, FY 2011-2002.

Port Everglades Waterborne Commerce Chart FY 2011-2002 (Unaudited, Revised 3/27/2012)											
	2011	2010	2009	2008	2007	2006	2005	2004	2003	2002	Averages
Total Ship Calls	4183	4079	4251	5226	5496	5510	5901	6389	5853	5484	5237.2
Container Ships	1861	1830	1980	2197	2270	2185	1988	1890	1880	1859	1994
Cargo Ships	180	113	105	157	202	268	247	231	213	196	191.2
Petrol. Tanker/Barge	630	661	683	727	732	744	751	763	798	748	723.7
Cruise Ships	969	1015	1007	1676	1852	1763	2362	2854	2215	1963	1767.6
Navy/USCG	26	29	34	22	39	29	18	25	17	22	26.1
Other bunkers/tugs	517	431	442	447	401	521	535	626	730	696	534.6

Commerce Chart for Port everglades between the years of 2002 and 2011. Each row represents a type of vessel that came in and docked at the port during the subsequent year in the column.

in Broward County. Approximately 5,237 commercial ships and 1,767 cruise ships berth at Port Everglades every year (Table 1). This number does not include the hundreds of

recreational vessels that are docked in or around the port. Depending on the size and mechanical upkeep of the vessels they can produce 1,300 to 5,300 gallons of bilge water every 24 hours (Johnson 2008). With an average of 3,300 gallons of bilge water entering the ocean per ship on any given day, the amount of oil and gasoline can be substantial. Because petroleum products entering the marine environment are seldom consistent, researchers use water-accommodated fractions (WAFs) as an effective means to test the effect of pollutants on organism. The WAFs are prepared mediums, derived from low energy mixing of oil or petroleum products with sea water, which is essentially free of bulk material particles (Singer 2000). The WAFs are considered to contain the highest possible concentration of dissolved petroleum hydrocarbons expected from oil spills (Faksness 2008).

1.2 Effects on invertebrate reproduction

Echinoids are good candidates as toxicological model organisms for several reasons, including their ecological relevance, benthic and relatively sedimentary lifestyle, and their high sensitivity to micropollutants (Zito *et al.* 2005; Bellas 2008; Nipper *et al.* 1997, Ozretic *et al.* 1998). Echinoids are dioecious and are broadcast spawners that release both egg and sperm into the water column to fertilize externally (Barnes 1987). Sea urchin eggs are encased in two layers of acellular coats, the jelly coat (a transparent, gelatinous outer layer consisting of globular glycoproteins bound to a fibrous fucan superstructure) *et al.* and the vitelline coat (extracellular protein matrix) (Bonnell *et al.* 1994). The sperm's head is covered by an organelle called the acrosome, which contains enzymes used to penetrate the egg (Hoshi 1994). During sea urchin fertilization, the sperm’s acrosome enzymes digest the egg’s jelly coat and vitelline coat. The proteins on the sperm head bind to the egg receptor and the plasma membrane of the sperm and the egg fuse. The sperm nucleus enters the egg cytoplasm, triggering the rising of the fertilization envelope and stopping other sperm from penetrating the egg and resulting in polyspermy (Kobayashi 1981).

Toxicity studies on the sperm specifically have been conducted since the 1960’s utilizing echinoid gametes. Warnau *et al.* (1966) studied spermiotoxicity in echinoids (*Paracentrotus lividus*) in the Mediterranean. The researchers studied the effects of sperm

exposure to fertilization rates using heavy metals including Cu, Ag, Cd, and Hg. High concentrations of heavy metals were toxic to sperm, and fertilization rates lowered with increased metal concentrations. Au *et al.* (2001) also showed the effects of cadmium having detrimental effects to *Authocidaris crassispina's* sperm; sperm mobility decreased, they postulated this could because by structural impairment due to chronic exposure to Cd^{+2} (Au *et al.* 2001, part 1 & 2). Oil and oil dispersants, such as Esso Corexit 9527, were also found to reduce fertilization ability of the spermatozoa in sea urchins with concentrations as low as 0.0003 ppm (Hagstrom and Looning 1977).

Oocytes are considered to contain numerous ways including multiple types of proteins, DNA repair and anti-oxidant enzymes of protecting against environmentally induced damages, (Aitken *et al.* 2004, Lewis and Galloway 2009). Kobayashi (1972, 1974, 1977, and 1981) studied Japanese sea urchin, *Anthocidaris crassispina,* and the Pacific sea urchin, *Strongylocentrotus droebachiensis*. Over the course of several studies, the eggs and embryos were exposed to several marine pollutants including heavy metals (Cu, Zn, Cd), and chemicals like ABS (alkyl benzyl sulphonate), ammonia (NH_3), Bunker C oil, and BP 110X oil dispersant, which had varying degrees of toxicity to the embryos. The order of toxicity for the heavy metals and chemicals was Cu > Zn > Cd > ABS > NH_3. The pollutants reduced fertilization and hindered development in the initial stages of cell division. Extensive numbers of arrested blastulae occurred, the higher the concentration the greater the embryonic retardation and cytolysis. The formation of pluteus was abnormal or retarded and there was no evidence of polyspermy at higher concentrations of pollutants (Kobayashi 1981). While these studies concentrated on echinoid eggs, there has been no examination of the egg morphological structure and most of the studies were concentrated on the influence of pollutants on zygotes. Research on petroleum products have demonstrated similar results as the above studies *Lytechinus anemesis* showed that polycyclic aromatic hydrocarbons (the toxic fraction of spilled petroleum) disrupted the development of the A/V axis patterning (β-catenin dependent pathway) in the exogastrulation (Pillai *et al.et al.*. 2003). Bellas *et al.* (2013) evaluated the toxicity of WAFs of artificially weathered standard fuel oil on mussel (*Mytilus galloprovincialis*) and sea urchin (*Paracentrotus lividus*) embryos over 80 days. The marine fuel oil was artificially weathered by stirring continuously with a magnetic stirrer,

which promotes dissolution and accommodation into an aqueous phase, leading to evaporation, biodegradation, and photolysis (decomposition of molecules by the action of light) and photooxidation (oxidation caused by the action of light) of the fuel oil. The results indicated that fuel oil that was weathered longer increased the toxicity up to eight fold to sea urchin embryos, where previously it was thought that weathering would reduce the potency of the pollutants. Nichol *et al.* (1977) studied the effect of crude and fuel oil WAFs on *Mellita quinquiesperforata* (sand dollar) fertilization and development. The ratio of sea water to oil used was 1:8 and the WAFs were mixed for a period of 24 hours. They observed egg morality, delays in first cleavage, abnormal cleavage, depressed respiration and sperm mobility, and reduced development. The results indicate that sub-lethal toxicity may contribute to poor development and low fertilization rates in the presence of these pollutants but further research is needed across contamination levels. Berdugo *et al.* (1977) studied the effects of WAFs on the reproduction of *Eurytemora affinis* (estuarine copepod). The researchers noted significant reduction in length of life, total number of eggs that could be produced; mean brood size and rate of egg production. When exposed to naphthalene alone, they noted a significant reduction in female fecundity. Fadlallah (1983) noted that coral gametogenesis, fertilization, and larvae metamorphosis are disrupted by exposure to petroleum products. Negri & Heyward (2000) found that chronic crude oil pollution on a Red Sea reef was shown to cause higher rates of coral colony mortality, fewer breeding colonies, a decrease in the number of ovaria per polyp, and a decrease in the average reproduction index. The authors suggest that their results indicate that fuel oil may be more toxic to fertilization than crude oil, but further studies are needed.

1.3 Ecological importance of echinoids

Echinoids, i.e., sea urchins, sea stars, sand dollars, are one of the most diverse and successful echinoderm groups. They are exclusively marine organisms and distributed throughout all the oceans and all depths (Rupper and Barnes 1994). Echinoids occupy keystone positions in many vulnerable ecosystems from the Artic to tropical regions (Byrne 1994). Benthic echinoid taxa are often important ecosystems engineers and bioturbators. Sea urchins are powerful herbivores and their grazing has a significant

effect on macroalgal abundance, productivity, and diversity on coral reefs (Bellwood 2004).

Sea urchins play a critical role in the health of their relative communities. For instance, increases in the formation of grazing fronts in kelp ecosystems can lead to a transformation of the biological community. Here, urchins are voracious, herbivorous mesopredators feeding on the kelp stalks, sea grasses and macroalgae (Gregor 2001). Without the predation (i.e., sea otters and fish) on their populations, sea urchins have been linked to massive destructions of kelp-forest habitat (Bernstien *et al.* 1981, Cowen 1983, Tegner and Levin 1983, Dean *et al.* 1984, Harrold and Reed 1985, Miller 1985, Scheibling and Hamm 1991). Sea urchins are however, susceptible to local and large-scale perturbations and reversals in community dynamics can occur quickly. Prior to 1983, the sea urchin, *Diadema antillarum,* was ubiquitous in Caribbean tropical and subtropical ecosystems and attained population densities of up to $21m^2$ (Carpenter 1988). In January of 1983, a massive mortality event began in Panama and circulated through the Caribbean. By February of 1984, more than 95-99% of the *D. antillarum* population died due to an unknown pathogen (Carpenter 1985, Levitan 1988).

The reefs during the *D. antillarum* die-off saw an increase of algal biomass by 20% in the beginning and to up to 50% towards the end of 1984 (Carpenter 1988). The loss of important herbivores combined (Lessios 1988) with deteriorating water quality from increased nutrient input, i.e., eutrophication (Lapointe 1997) have led to a phase shift from coral to macroalgae dominated reefs (Mumby 2009). This phase shift from scleractinian corals to macroalgal dominance is difficult to reverse because of complex ecological processes (McManus and Polsenberg 2004). *Diadema antillarum* have been slow to recover (Bellwood 2004), but as they recover at sites in Jamaica the benefits are evident by increased juvenile coral density (Edmunds and Carpenter 2001). In cage studies in St. Croix, *D. antillarum* grazing is found to control biomass, species composition, and the metabolism of many coral red algal turf communities (Carpenter 1985). Further loss of sea urchins in subtropical and tropical ecosystems would be devastating to coral reefs and adjacent seagrass beds.

1.4 Purpose

Previous studies have focused on accidental crude oil spills and subsequent dispersant use on broadcast spawning marine invertebrates. What remains unclear is how petroleum pollutants from daily release affect echinoid reproduction. This research focuses on the effects of how petroleum pollutants damage echinoid eggs and how this damage can influence fertilization and larval viability.

1.5 Objectives

The objectives of this project are to understand the impacts of oil and gas WAFs on fertilization success in two sea urchin species. The project will examine:

1. if the integrity of the outer egg layer has been compromised by oil and/or gas.
   H1: The legal amount of oil/gas allowed to be discharged into the ocean will damage the egg's outer layer.
2. if the integrity of the outer egg layer is compromised by pollutants, is fertilization effected.
   H1: Fertilization will decrease with increased petroleum concentration.
3. if petroleum pollutants influence embryo viability after 48 hours of exposure.
   H1: With increased concentrations of WAFs, viability of the larvae will decrease linearly.

Chapter 2

2.1 Introduction

The detrimental effects of petroleum pollutants to the marine ecosystems have made worldwide headlines in recent years. The Deepwater Horizon oil spill caused 210 million gallons of oil to escape into the Gulf of Mexico and approximately 1.84 million gallons of dispersants were then applied to dissipate the oil (Cleveland 2010, Dept. of Interior 2012). Clean-up efforts successfully captured 33.6 million gallons of oil but most of the oil remains unaccounted for (Dorsett 2010). While oil spills like the Deepwater Horizon have received high priority because of their national exposure and large quantity, petroleum pollutants that are introduced daily are not widely discussed (McCook 1999, Nystrom *et al.* 2000, Bellwood *et al.* 2004). Evidence suggests that of the 3.5 million tons of oil that is released into the ocean every year, only a very small percent is attributed to oil tanker spillage (Wiggins *et al.* 2000). Close to 70% of oil pollution is due to chronic pollution from the dumping of petroleum waste as bilge water and municipal and industrial runoff (Wiggins *et al.* 2000).

Bilge water is the mixture of water, oily fluids, lubricants, gasoline, cleaning fluids and other similar wastes that accumulate in the lowest part of a vessel. The amount of bilge water in a ship is highly variable and dependent on factors including the size of the ship, engine room design, preventive maintenance, and the age of the components (US EPA 1999). Bilge water has to be pumped out of the ship, thus it is one of the main sources of oil in the marine environment. The International Maritime Organization (IMO) and the EPA specify that bilge water may be discharged within 12 nautical miles of the coast only if its residual oil content is equal to or less than 15 parts per million (ppm) per gallon. The EPA does not restrict bilge release outside of 12 nautical miles from the coast (Wiggins *et al.* 2000).

Scientists have long been concerned about the detrimental impact of petroleum pollutants on marine organisms (Allen 1971, Moore and Dwyer 1974, Berdugo 1977, Kobayashi 1981, Teal 1984, Negri and Heyward 2000, Soysa *et al.* 2012). Since petroleum products entering the marine environment are seldom consistent, researchers who test the toxicity of crude or fuel oil contamination use water-accommodated

fractions (WAFs) as an effective way of controlling the pollutant amounts in an experiment. The WAFs are prepared mediums, derived from low energy mixing of oil or petroleum products with sea water, which is essentially free of particles of bulk pollutants (Singer 2000).

Echinoids (sea urchins) are good candidates for toxicological studies because of their ecological relevance, benthic and relatively sedimentary lifestyle, rapid response and high sensitivity too many types of contaminants, including micropollutants stored in marine sediments (Zito *et al.* 2005; Bellas 2008). Spawning, or the releasing of gametes into the ocean, is the most common method of reproduction for echinoids (Giese and Kanatani 1987). During spawning the gametes and resulting larvae are at risk to pollutant exposure (Bellas 2013); therefore it is important to understand the effects of petroleum products on echinoid reproduction, development, and larval survival. While many studies have concentrated on the effects of petroleum products on sperm and fertilization (Koyayshi *et al.*. 1980, Kobayashi 1981, Nipper *et al.* 1993), little is known about how eggs are affected. Here, I examined the major components of bilge water on the reproductive success of two echinoid species. The objectives of the project were to determine if the integrity of the egg layer has been compromised by petroleum pollutants, and how this affects fertilization and larval viability after 48 hours.

2.2 Methodology

2.2.1 Species Description

Lytechinus variegatus, commonly called the green variegated sea urchin, belongs to the Class Echinoidea (Keir 1974, Smith 1984). It is the largest species in its genus, with the test reaching 92 mm in diameter (Watts 2001); the test may be purple, green or dull red, blotched with white. Their spines are mostly short and vary in color (Humann 1992). *Lytechinus variegatus* is found in a wide variety of habitats including coral reefs, seagrass beds, mangrove roots, sand and in the back reef and reef flat environments (Yender and Michel 2010, Ernest and Blake 1981, Pena *et al.*., 2008). This species is found along the southeastern coast of the United States and Caribbean and often harvested as a delicacy (Moore *et al.*. 1963, Wolcott and Messing 2005). Physical variables, like temperature, salinity (Moore 1966), and pH (Chapman 1995) are factors

that influence the timing, intensity, and duration of reproductive events in echinoids (Ernest 1981). Other variables like seasonality, sperm concentrations, and phytoplankton biomass also contribute to the reproductive cycles of echinoids, specifically *Lytechinus variegatus* (Rueter and Levitan 2010). With the warm weather in South Florida, these echinoderms can spawn year round and in semilunar patterns (Moore *et al.* 1963). Although spawning in *L. variegatus* typically peaks from May to July (Pena *et al.*. 2008, Tennent 1910), individuals collected in Southeast Florida can be induced to spawn in December (Fogarty pers. obs.) and January (Pelikan per obs.).

Eucidaris tribuloides, commonly called the slate pencil urchin, is a cidaroid sea urchin that inhabits littoral regions of the Atlantic Ocean. Cidaroida is an order of primitive sea urchins, the only living order of the subclass Perischoechinoidea. All other orders of this subclass, which were even more primitive than the living forms, became extinct during the Mesozoic Era (Kroh and Hansson 2014). Cidaroids are morphological, developmental, and molecularly unique, and considered primitive because the embryos have no mesenchyme cells ingress before gastrulation, yet larvae still contain calcium carbonate skeletons, like more derived sea urchins (Schroeder 1981, Wray and McClay 1988). *Eucidaris tribuloides* can be found up to 800m, although it is most commonly found 0-50m (McPherson 1968). The test is light to reddish-brown; the large, blunt primary spines are usually light brown, striped with darker brown, or even marbled with white, often tinged with green or red (Humann 1992). The spines are often encrusted with algae, bryozoans or even sponges. *Eucidaris tribuloides* can be found on reefs and rubble areas and often hidden inside small recesses. The test and spine size grows up to 6 cm (Lamark 1816). Reproduction seems to be sensitive to both seasonal and lunar cycles. In the Florida Keys, *E. tribuloides* spawning in late summer and early fall while populations in Panama are found to spawn in spring, summer, and fall around the full moon (Lessios 1991).

2.2.2 Collection

Lytechinus variegatus were collected at Blue Heron Bridge, Riviera, Florida and in the Florida Keys (24°37'18.71"N, 81°24'8.13"W). *Eucidaris tribuloides* was collected on the east side of Blue Heron Bridge, in Riviera, Florida. Several trips were necessary to collect enough individuals to conduct the experiments, with 20-40 sea urchins needed for

each run. Sea urchins were collected while scuba diving transported to the laboratory in coolers full of sea water with battery powered aeration.

Sea urchins were kept in cages in the Nova Southeastern University Oceanographic Center's boat basin. These cages were attached to floating docks with rope, and secured one to two feet above the ocean floor during low tide. Sea urchins were kept in the boat basin because previous attempts to keep them healthy in laboratory aquaria failed. The urchins were fed carrots and cabbage one to two times per week. The cages were also scrubbed once a week to reduce fouling. After each experiment, all sea urchins were returned to the sites from which they were collected.

2.2.3 Water-Accommodated Fraction preparation

The various treatments were made using the Standardization of the Preparation and Quantitation of Water-accommodated Fractions (WAF) of Petroleum for Toxicity Testing guidelines by Singer *et al.* (2000). Oil was purchased at a local boat shop, Quick Silver Marine Lubricants: Sterndrive and Inboard, 4-cycle engine oil, SAE 25W-40. The 90 octane non-ethanol gasoline was obtained locally from Hollywood Marina and Harbour Towne Marina.

The WAFs were prepared in 5L aspirator bottles designated specifically for this type of experiment. Each aspirator was setup on a magnetic stir plate, and filled with 1L of FSW and a specific amount of oil, gas or both depending on the treatment. Each sample was mixed at 400-500rpm to maintain a consistent mixing of the treatment water for 24-36 hours before the experiment was conducted.

A variety of treatment concentrations of each medium (oil, gas, and oil + gas) were used: control = 0 ppm, low concentration = 15 and 30ppm, moderate concentration = 60 and 100ppm, high concentration = 500 and 1000ppm. With the EPA's legal limit for petroleum products in bilge water being 15 ppm per gallon within 12 nautical miles of the coast, it can be a challenge to gauge the amount of petroleum products released in high boat traffic areas.

2.2.4 Induced Fertilization

Spawning was induced by injecting each sea urchin with 1-3 ml of 0.55M KCl (potassium chloride), with an average of 2-3 ml dependent on test size. The urchins were injected on the oral side, near the Aristotle lantern with a 22 gauge syringe. Sperm, identified by whitish color was forcibly extruded through the five gonopores on the aboral surface. The dry sperm was collected with Pasteur pipettes and transferred to a watch glass and placed on ice. Females, identified by the orange gelatinous eggs they extrude, were inverted and placed in a large petri dish with filtered sea water (0.2 µm). The eggs and sea water was placed in Erlenmeyer flasks and used as egg stock.
Sperm stock was created for each of the males by adding approximately 250 µl of dry sperm to 9.9 ml of FSW and 0.5 ml of the stock was preserved with Z-fix (buffered zinc formalin fixative) for sperm counts using eight replicate counts on a hemacytomer. Test sizes were measured using calipers.

Each 20ml glass experimental vial was filled with 8 ml of WAF or FSW(control), followed by 1 ml of egg stock solution, and 1 ml of sperm stock. Vials were then swirled three times and left undisturbed for two hours. At that time the number of embryos with raised fertilization envelopes and the number of unfertilized eggs were quantified using a compound microscope. After scoring fertilization, approximately 100 embryos were placed in mason jars filled with 300ml of FSW for 48 hours to examine early development.

A second experiment was conducted to determine the impact of prolonged petroleum exposure on eggs. Here, 1 ml of egg stock was added 8 ml of WAF or FSW (control) for 2 hours prior to fertilization. A subset of eggs, (0.25 ml), from the egg stock and treatment was preserved using 3% glutaraldehyde with 0.2 M sodium cacodylate (pH 7.4) solution (Schatt and Feral 1996) for later scanning electron microscopy (SEM) analysis. After the SEM samples were collected, 1 ml of sperm stock was introduced to the experimental vials and swirled three times. Fertilization was scored and embryos were placed in jars as stated above.

2.2.5 Development and Larvae Viability

After scoring fertilization, the quantified number of embryos and unfertilized eggs were added to a large (16oz) mason jar for culture. A mixture of penicillin and streptomycin antibiotics (6,000mg/ml and 10mg/ml respectively) was added to the cultures to reduce the bacterial accumulation from decaying eggs (per Strathman 1987). After 48 hours, approximately 280 ml of water was removed by siphoning the water through a 100 micron nitex mesh in order to concentrate the larvae. The number of healthy, normally developing larvae and the number of abnormal larvae were quantified. Healthy, normally developed embryos were at the gastrula or pluteus stages.

2.2.6 Scanning electron microscope (SEM)

Scanning electron microscopy was used to determine if the integrity of the jelly coat and vitelline layer had been compromised by the petroleum products. To prepare the eggs for SEM, multiple solutions of ethanol (EtOH) were used to dry the eggs. The eggs were placed in multiple solutions of EtOH (20%, 50%, 75%, 90%, and 100%) for 5 minute increments; this was repeated in triplicate for each solution. Once the eggs were in 100% solution, the eggs were then transported under the hood for the final stages of the drying. Finally three Hexamethyldisilazane (HMDS) solution changes occurred at 5 minutes each. The eggs were then pipetted onto thin circular slip covers (12mm), held with forceps until dried and then placed on SEM stubs using carbon adhesive tabs. Circular slip covers were used after poor results with square slip covers that caused samples to clump. Once attached, the stubs were placed in the hood and left to off-gas overnight.

Sputter coating is the standard method for preparing non-conducting or poorly conducting specimens for observation in SEM. Sputter coating is a process that covers the specimen with a thin layer of conducting materials, such as gold/palladium alloy. A conductive coating prevents charging of the specimen with an electron beam during SEM analysis. Each sample is run through the sputter coater two to three times to coat thoroughly. We initially coated samples once as recommended in the literature, with significant charging occurring. Once properly coated, samples were placed in the SEM and 10-50 images were collected for each sample.

The scanning electron microscopy images were analyzed using two a qualitative and semi-quantitative metrics. The magnitude of damage sustained was categorized qualitative on a scale from 1 to 5, with 1= normal eggs and 5=severely degraded eggs. Additionally, the SEM images were placed in 6 categories: 1= normal/undamaged egg, 2=misshaped eggs, eggs that are no longer spherical, 3=holes in the different egg layers, 4= sieving or sloughing egg layers, 5= shrunken eggs, 6= sieving and holes in egg layers.

2.2.7 Statistical analysis

Statistical analyses for these experiments were conducted using JMP 11 and 12 software (SAS: Cary, N.C.). When the necessary assumptions were met, the proportion of fertilized eggs for each treatment concentration was analyzed using an ANCOVA with log sperm concentration as the covariate to determine if variance in fertilization could be explained by sperm density. Because polyspermy can also cause latent developmental failure, an ANCOVA was also used to examine larval viability. To directly compare the pollutant concentrations to the controls, I subtracted the treatment from the control to obtain a number that ranged from -1 to 1. A value close to zero indicates there was no difference between controls and treatments, while a value close to 1 indicates that the pollutants lowered fertilization. If the appropriate assumptions were met, a one-way ANOVA was used to determine differences across concentrations within each treatment (gas, oil, gas + oil). A nonparametric Kruskal-Wallis test was used if assumptions were not met. Development was categorized by stage: pluteus, gastrula, and abnormal development and were compared by treatment type and experiment using chi square. The semi-quantitative SEM images were analyzed with a Kruskal-Wallis test.

2.3 Results

Species: *Lytechinus variegatus*

2.3.1 Demographic Data

The sex ratio was skewed towards males in all trials (Appendix: A.2). The average test size for males was 59mm, with the largest recorded in April with a max of 78mm and the smallest recorded in September with a minimum test size of 43mm. The average test size for females was 60mm, with the largest recorded in April with a max of 81mm and the smallest recorded in September with a minimum test size of 43mm (Appendix: A.4). There is a statistical difference in the test sizes; the first three trials (January, April, and July) were significantly larger (ANOVA $p<0.001$) than the trials at the end of the year (September, October, and December). The highest proportion of spawning individuals was the end of September with 100% of injected individuals spawned (Appendix: A.6).

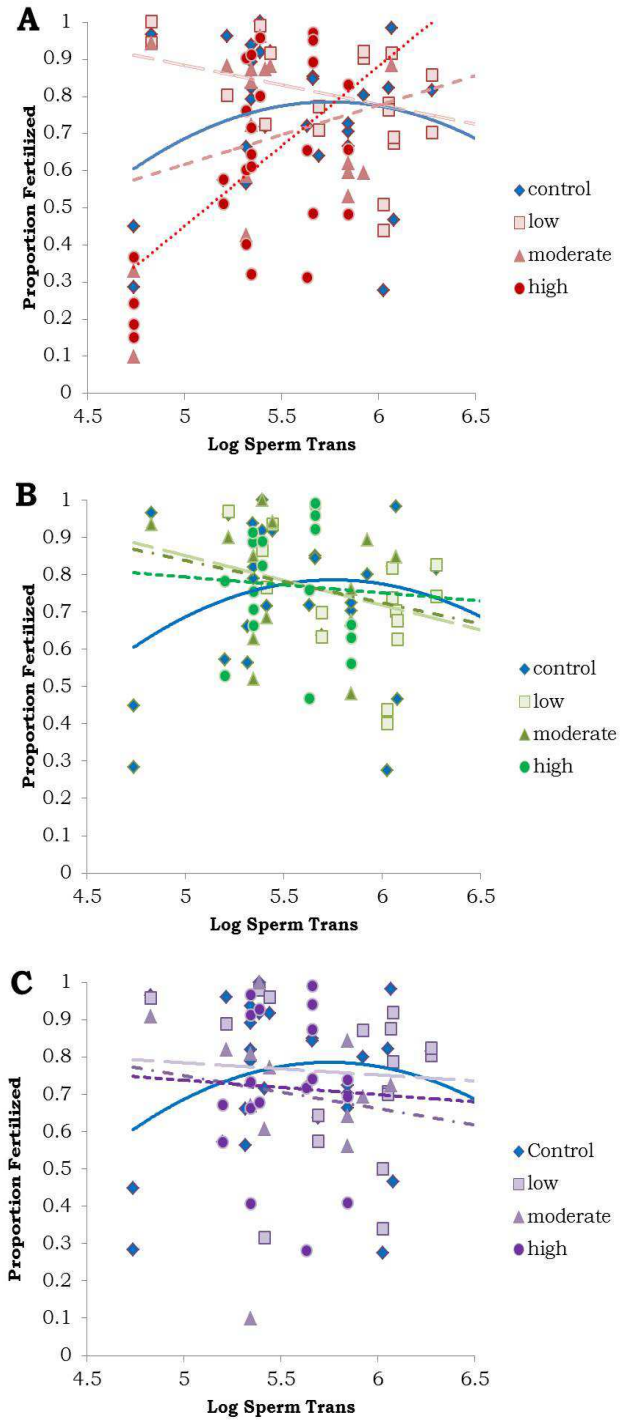


Figure 2.1 The proportion of *Lytechinus variegatus* eggs fertilized in experiment 1 as a function of log sperm among three treatments. A) Oil treatment, B) Gas treatment, and C) Oil + Gas treatment

source: gas	df	type III SS	ms	f	p
log sperm (linear)	1	0.07686657	0.07686657	1.4925	0.2256
log sperm*log sperm	1	0.00591242	0.00591242	0.1203	0.7297
treatment: Gas	1	0.01501254	0.01501254	0.2915	0.5908
treatment*log sperm	1	0.18359806	0.18359806	3.7357	0.0571
treatment*log sperm*log sperm	1	0.15065231	0.15065231	3.0653	0.0842
error	76	3.9142565	0.051503		
total	78	4.0000177		arc sin prop fert	

source: oil	df	type III SS	ms	f	p
log sperm (linear)	1	0.40930981	0.40930981	6.5982	0.0119
log sperm*log sperm	1	0.38748426	0.38748426	6.2464	0.0143
treatment: Oil	1	0.11243932	0.11243932	1.8126	0.1817
treatment*log sperm	1	0.05508314	0.05508314	0.8791	0.3511
treatment*log sperm*log sperm	1	0.01997226	0.01997226	0.3188	0.5738
error	87	5.3969323	0.062034		
total	90	6.3156725		arc sin prop fert	

source: oil & gas	df	type III SS	ms	f	p
log sperm (linear)	1	0.00000619	0.00000619	0.0001	0.9922
log sperm*log sperm	1	0.0197487	0.0197487	0.3139	0.577
treatment: Oil & Gas	1	0.0115735	0.0115735	0.1814	0.6714
treatment*log sperm	1	0.04452962	0.04452962	0.7079	0.4029
treatment*log sperm*log sperm	1	0.20410278	0.20410278	3.2445	0.0758
error	76	4.8484368	0.063795		
total	78	4.8600861			

Table 2.1 ANCOVA results of fertilization crosses for each treatment. The dependent variable is the proportion of eggs fertilized (Gas and Oil were arcsine-transformed.) The model consists of treatment group (treatments) as the main effect, with sperm per milliliter (logistic transformation as the covariate.

2.3.2 Fertilization

In the first experiment, the concentration of oil (ANOVA, p = 0.066) or gas (ANOVA, p = 0.160) did no significantly influence the proportion of eggs fertilized, yet when these pollutants were combined, the controls were significantly higher in the high concentrations compared to the low concentration [F(2,47) 4.047, p=0.024, Fig 2.2]. In the second experiment where eggs were exposed to oil pollutants for an extended period of time, concentration levels did not differ significantly (ANOVA, p=0.234). When eggs were exposed to gas, fertilization in controls was significantly greater in the high

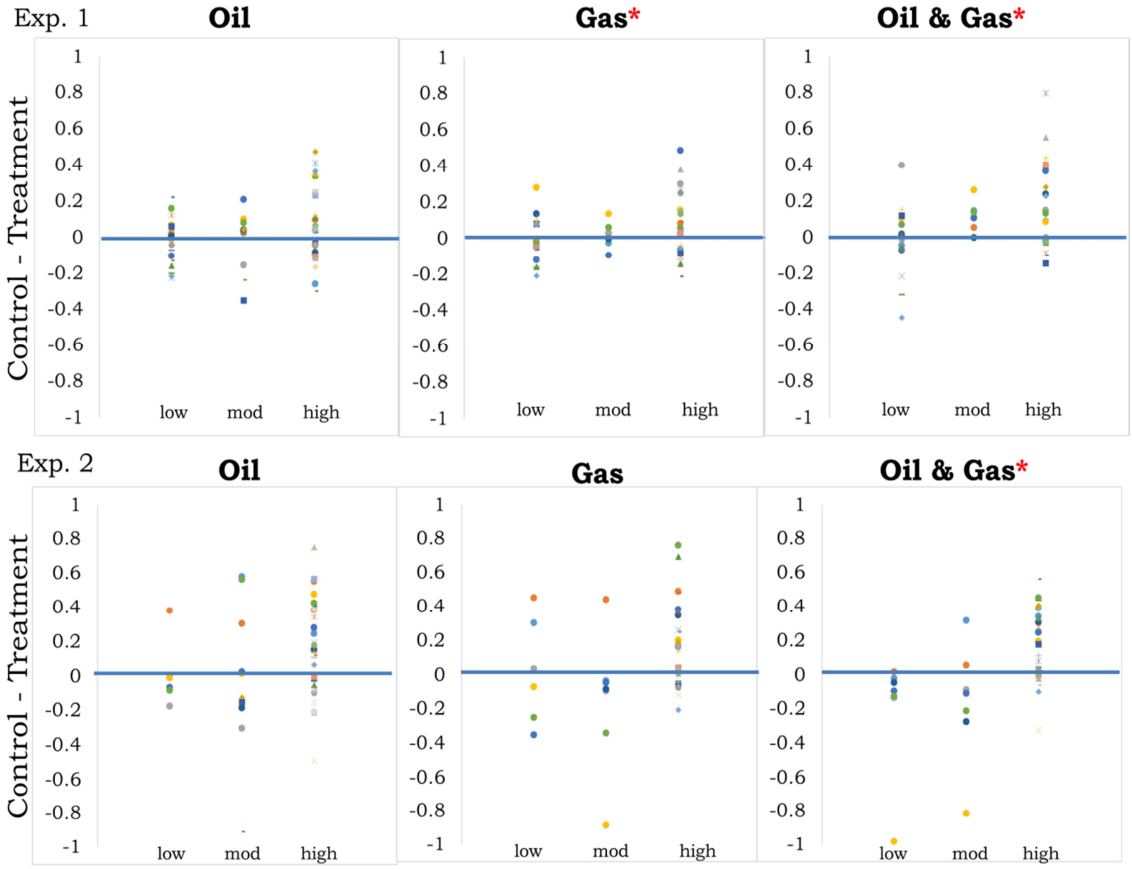


Figure 2.2 *Lytechinus variegatus.* Points denote the difference in controls and treatments across concentrations. Expected values were calculated based on proportion fertilized. Positive values denote higher fertilization in the controls than the treatment, while negative values denoted trials where treatments fertilization was higher than the control. Asterisk indicates statistical significance ($p<0.05$)

concentration compared to the moderate and low concentrations [F(2,36) =3.947, p=0.028, Fig 2.2]. For oil and gas combined, fertilization was higher in the controls in the high concentration than the low [F(2,37) 8.309, p=0.001, Fig 2.2] and moderate concentrations.

When taking into account the effect of sperm concentration on fertilization, there was no difference between concentrations for each treatment. In the first experiment with oil (Fig 2.1) the covariate was significant for a linear and polynomial fit (Table 2.1).

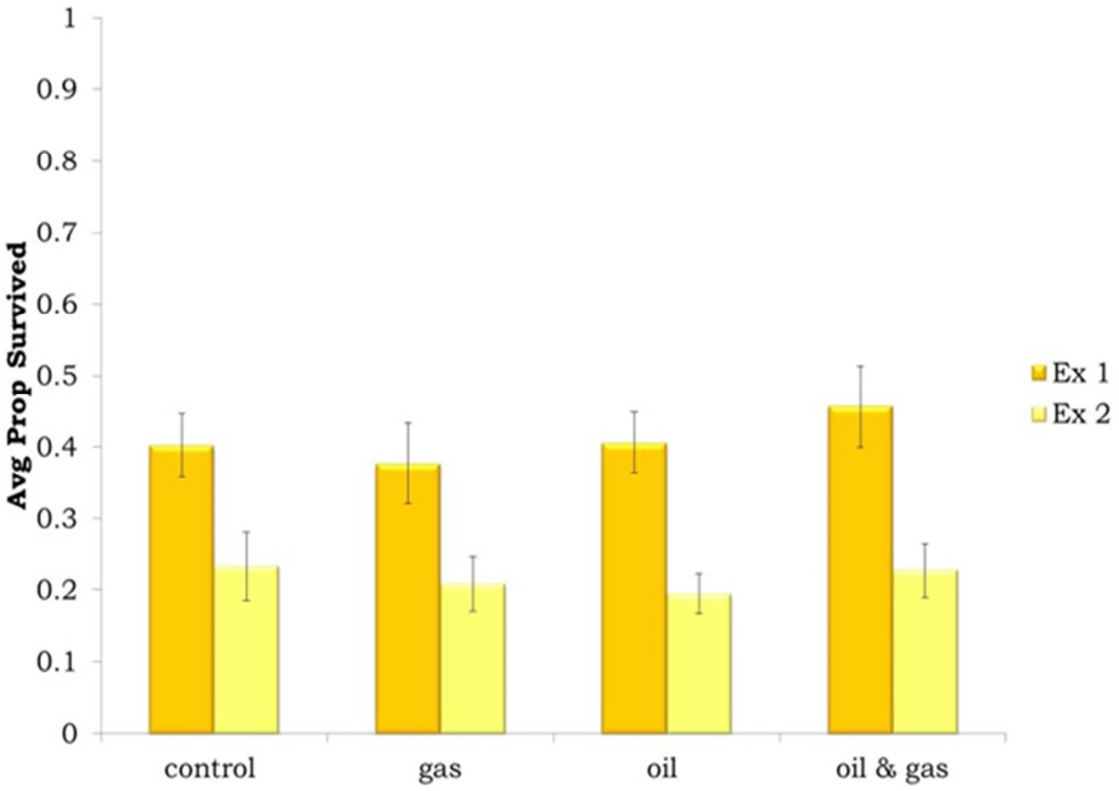


Figure 2.3 Comparing the average proportion of Lytechinus variegatus eggs that survived to 48 hours for both experiments. Kruskal-Wallis Test, $p < 0.0001$, standard error bars

2.3.3 Larval Viability

Larval viability differed between the experiments. . In experiment 1 oil treatment, the high concentration had significantly reduced survivorship than the low concentration, but not the moderate or the control for all three treatments [oil: F(3,45) =4.276, p=0.0097; gas: F(3,33) =3.171, p=0.037; oil + gas: F(3,33) =4.394, p=0.010, Fig. 2.4]. For experiment 2, the egg dosing experiment had significantly reduced larval survivorship compared to experiment 1(Kruskal-Wallis p<0.0001, Fig 2.3). Since the

data for experiment 2 could not be normalized, a Kruskal-Wallis test was performed to compare the concentrations. In the oil treatment, the high concentration was lower than the moderate concentration (H(2)=8.0368, p=0.0180, Fig.2.5A). In the gas treatment, the high concentration was significantly lower than the low and moderate concentrations (H(2)=7.4785, p=0.0238, Fig 2.5B). When the pollutant treatments were combined, there was no statistical significance (p=0.170).

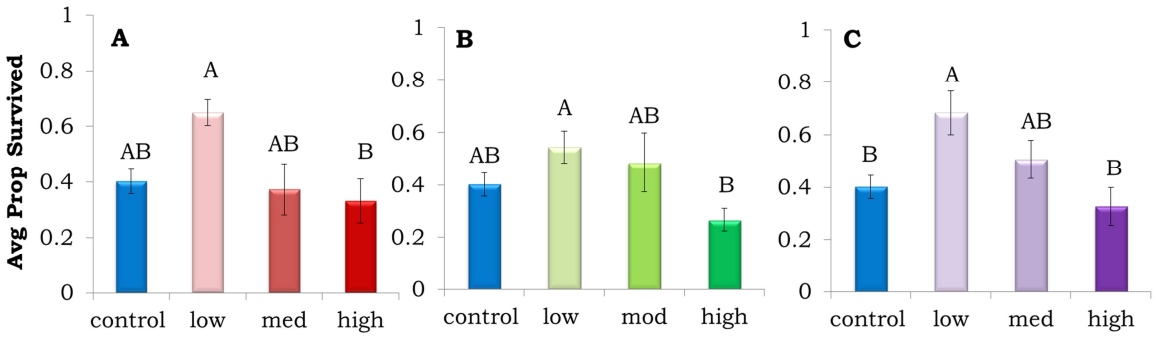


Figure 2.4 The proportion of *Lytechinus variegatus* eggs in experiment 1 that survived to 48 hrs. Statistical significance, A) Oil (p = 0.0097), B) Gas (p = 0.0371), C) Oil + Gas (p = 0.0104) with error bars with standard error. Different letter represent statistical significance based on Tukey's test.

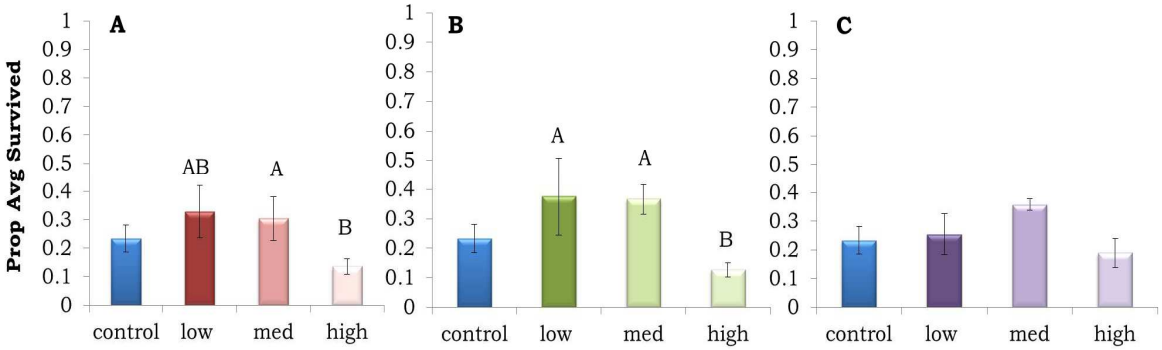


Figure 2.5 The proportion of *Lytechinus variegatus* eggs in egg dosing experiment that survived to 48 hours. Kruskal-Wallis test, A) Oil (p=0.0180), B) Gas (p=0.0238), C) Oil + Gas (p=0.1703) with standard error bars. Different letters represent statistical significance based on Tukey's test.

When comparing the developmental stage among the two experiments, after 24 hours there was significantly more pluteus that survived in the first than the second experiment. In both experiments, the pluteus stage dominated in all treatments

(H(2)=14.540, p=0.001, Fig.2.6) by experiment. In each experiment, there was a greater proportion of larvae in the pluteus stage than in either gastrula or abnormal development stage (experiment 1 [F(7,85) =213.965, p<0.0001,Fig. 2.7A] and experiment 2 [F(5,230) =4.063, p=0.002, Fig. 2.7B]).

2.3.4 Scanning Electron microscopy

The SEM images allowed the eggs to be categorized from normal to highly damaged. The controls had the highest proportion of normal, undamaged eggs, while all the treatments had the most damaged eggs (H(2)=28.636, p=<0.0001, Fig. 2.8). Categories based on type of damage sustained showed there were significant differences between the treatments: gas [F(5, 290)=8.518, p<0.0001)], oil

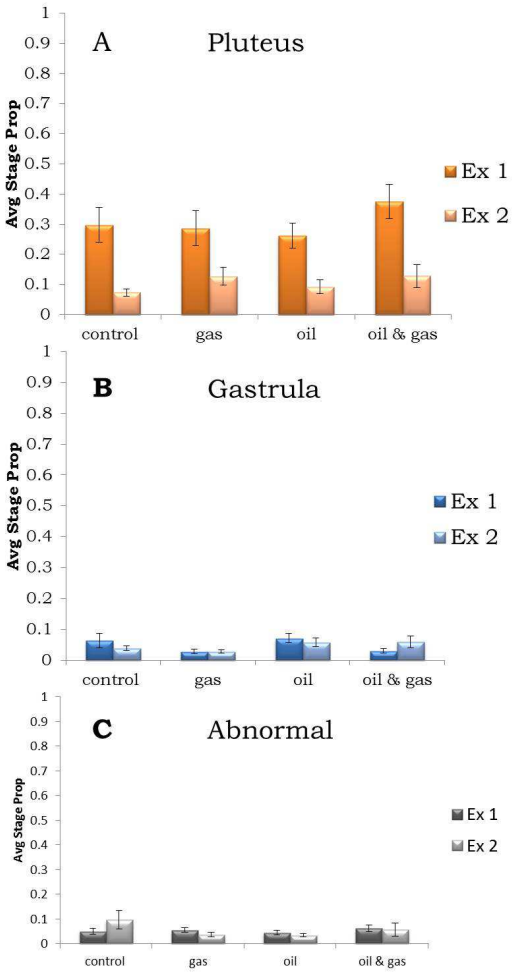


Figure 2.6 Comparing the development between the two experiments for *Lytechinus variegatus*, using nonparametric Kruskal-Wallis p = 0.0007

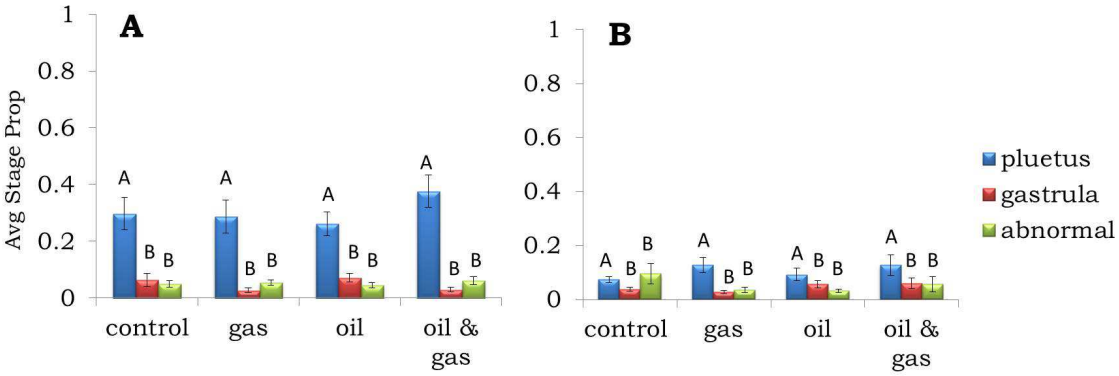


Figure 2.7 The average proportion of *Lytechinus variegatus* larvae at different developmental stages in the (A) first and (B) egg dosing experiment, statistical significance of p<0.0001 and p – 0.0015 respectively, with standard error bars

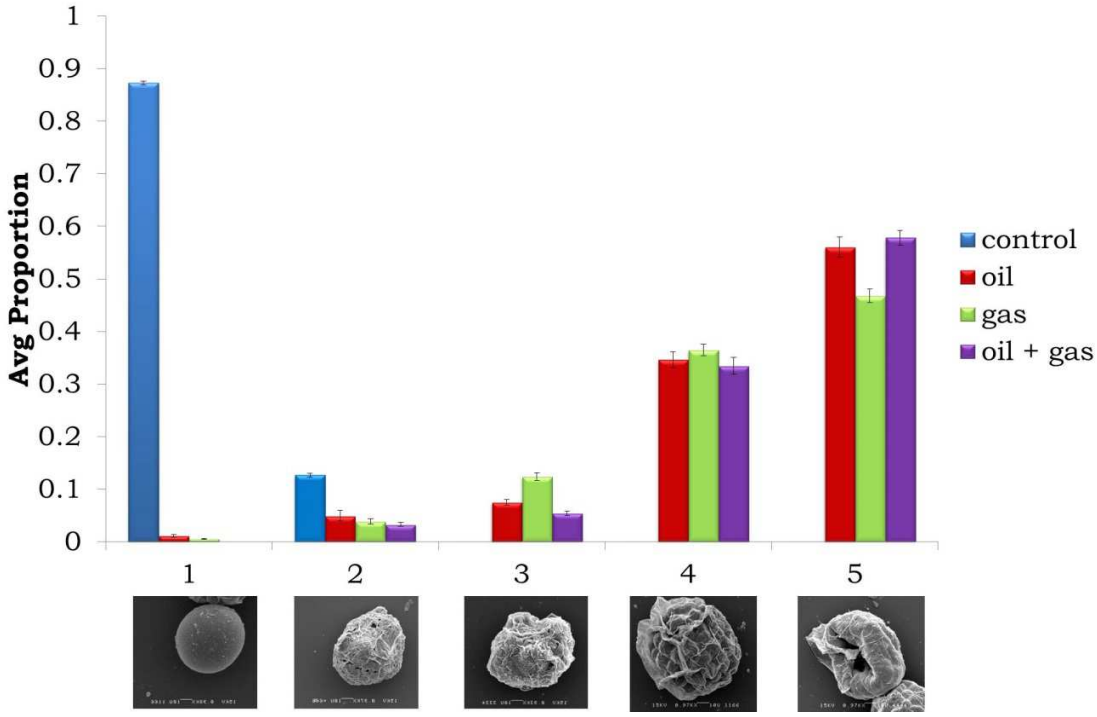


Figure 2.8 *Lytechinus variegatus* eggs quantified by the magnitude of damage sustained, 1=normal eggs while and 5= severly abnormal eggs. Wilcox test with statistical significance of p <0.00001, error bars with standard error.

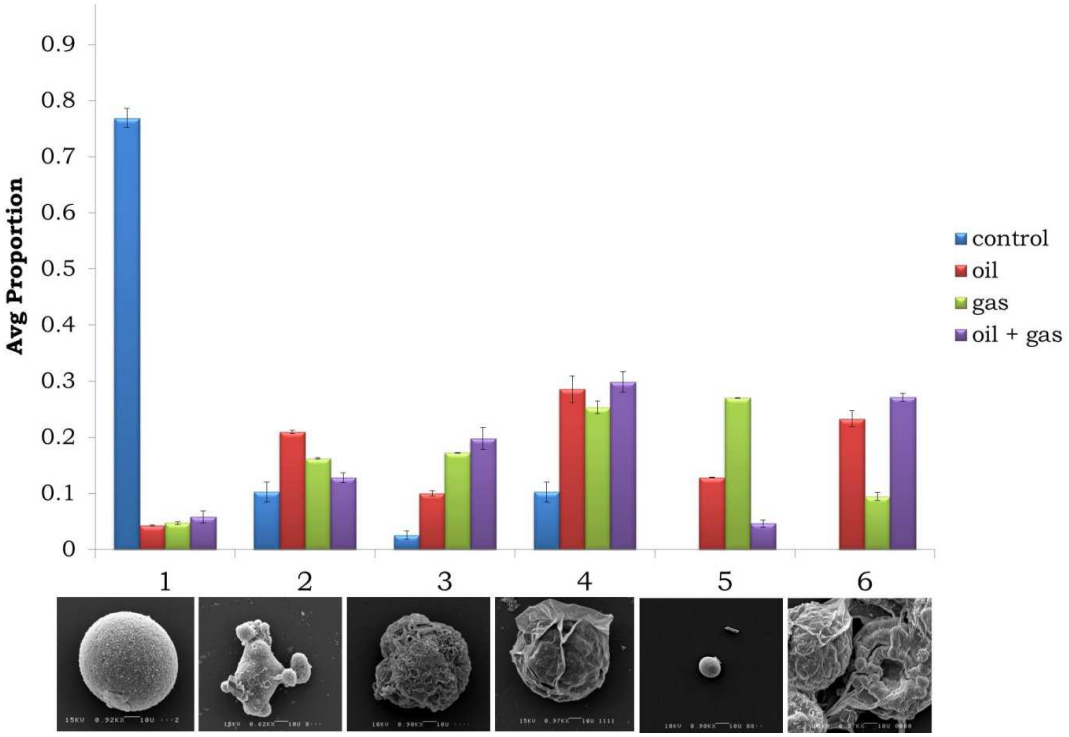


Figure 2.9 *Lytechinus variegatus* eggs categorized by damage sustained, 1= normal, 2=misshaped, 3=holey, 4= sieving layers, 5= shrunken, 6= holey and sieving. Statistical significance by Tukey's pair ise test of p=0.0009 (oil and gas) and p<0.0001(oil and gas), with error bars with standard error.

[F(5,204)=12.164, p<0.0001,Fig. 11)], oil + gas [F(5, 252)=4.296, p=0.001,Fig. 2.9)]. The controls had the highest proportion of eggs in category 1 (normal). The gas treatment had a significantly higher proportion of shrunken eggs (category 5). Oil and oil + gas caused more holes and sieving (category 6) eggs.

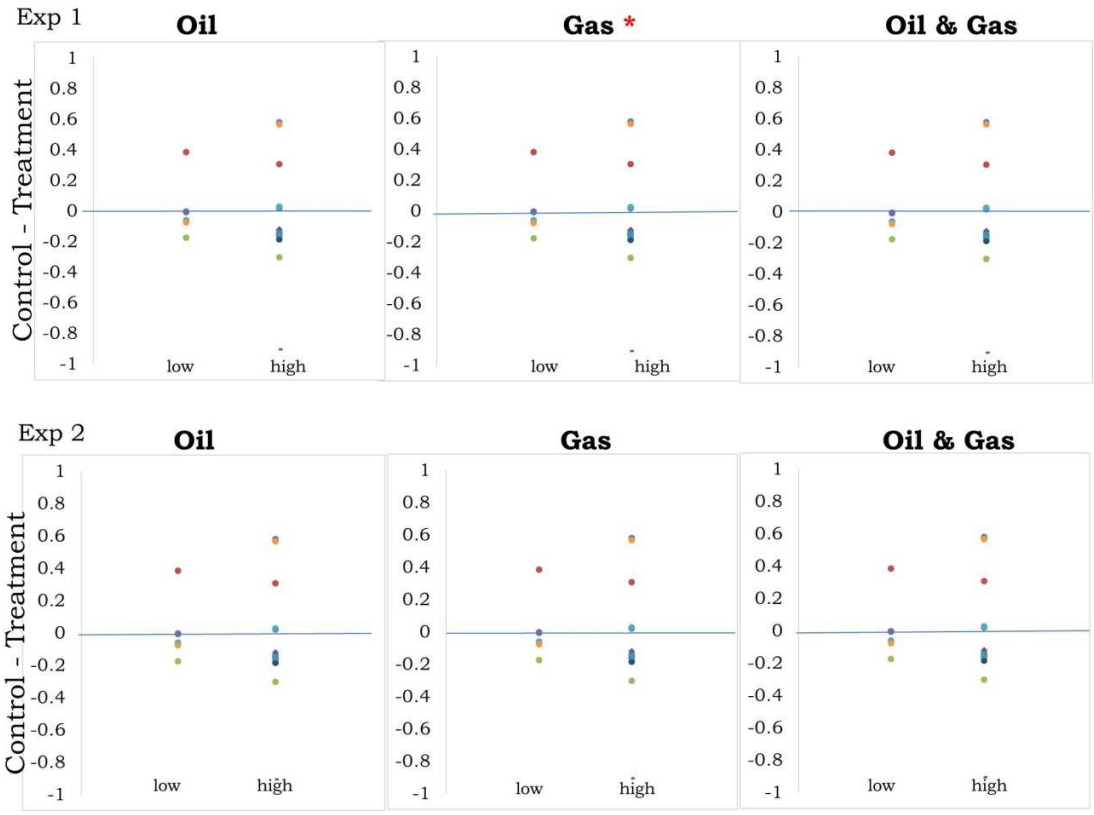


Figure 2.10 ***Eucidaris tribuloides*****.** Points denote the difference in controls and treatments across concentrations. Expected values were calculated based on proportion fertilized. Positive values denote higher fertilization in the controls than the treatment, while negative values denotes trials where treatment fertilization was higher than the control. Asterisk indicates statistical significance ($p < 0.05$).

2.4 Results, Species: *Eucidaris tribuloides*

2.4.1 Demographic Data

The sex ratio of *E. tribuloides* varied between the two experiments. There were more males in March than January (Appendix: A.3). The average test size for males was 28 mm, with the largest recorded in January with a max of 33 mm and the smallest recorded also in January with a minimum test size of 24 mm. The average test size for females was 30 mm, with the largest test recorded in January (35 mm) and the smallest recorded in March (26 mm, Appendix: A.5). This species had a low spawning rate with

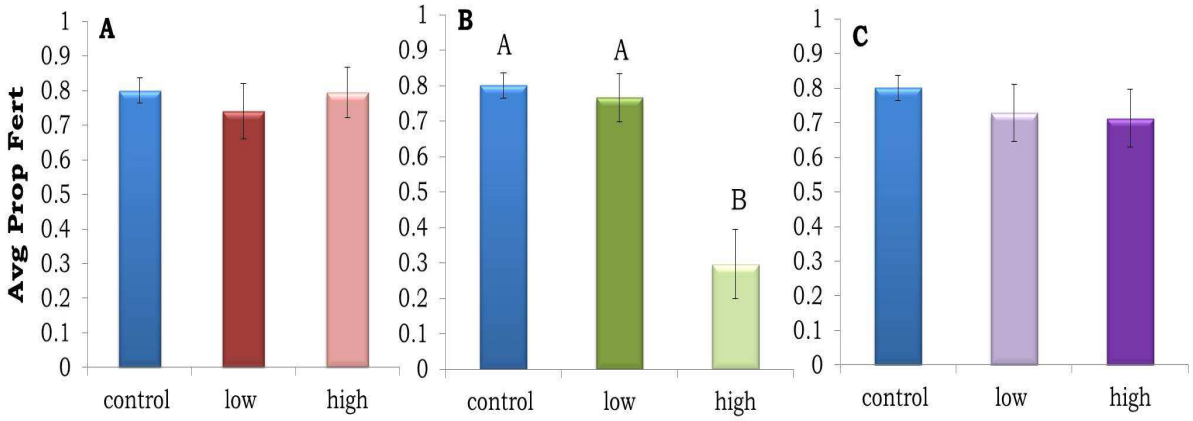


Figure 2.11 The proportion of *Eucidaris tribuloides* fertilized eggs in experiment 1 with standard error bars. ANOVA p=0.00025 for the gas treatment (B), letters represent statistical significance from Tukey's test. No statistical significance in the oil (A) or oil + gas (B) treatments.

14% spawning and no mortality during the trial. Mortality was checked on a daily basis after the first week the experiment was conducted (Appendix: A.7)

2.4.2 Fertilization

Sperm concentration did not significantly influence fertilization in either experiment with *E. tribuloides.* Fertilization in the controls were significantly higher than fertilization in the high gas treatment [F(1,14) = 12.6657, p=0.0031, Fig 2.11B and 2.10], while no significant difference was see among concentrations in oil or oil + gas (Fig. 2.10). In experiment 2, fertilization in the controls was significantly higher than the low

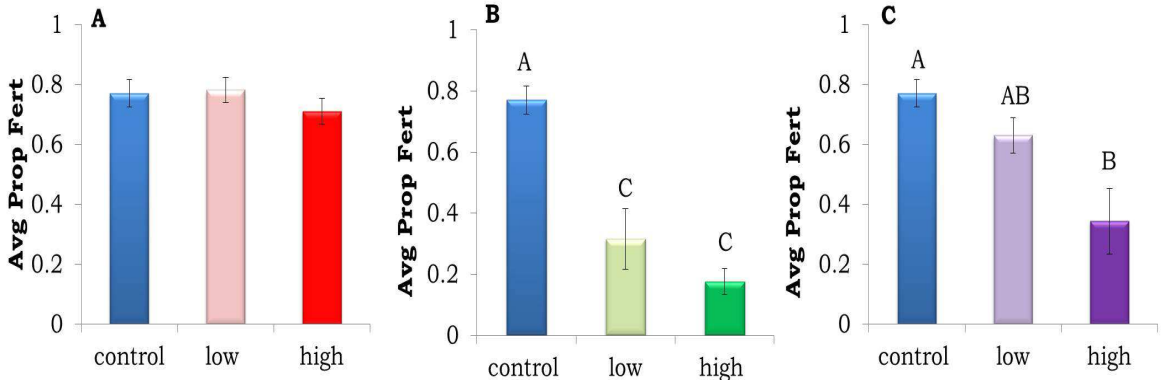
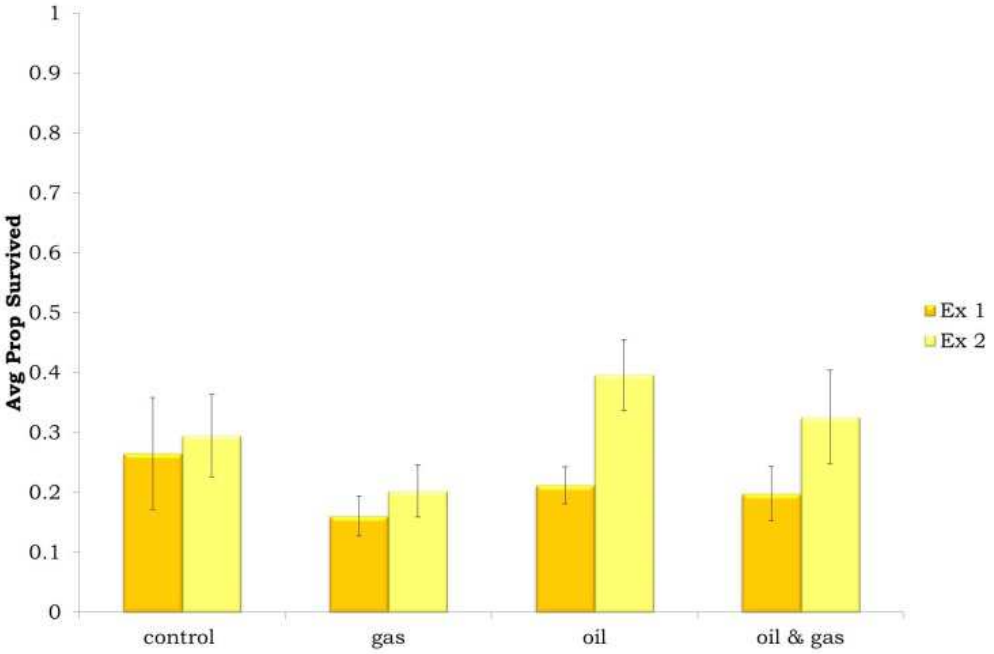


Figure 2.12 The proportion of Eucidaris tribuloides fertilized in experiment 2 with standard error bars. ANOVA p<0.0001 for the gas treatment (B) and ANOVA p<0.0001 for the oil +gas treatment (C)



FFigure 2.13 The average proportion of Eucidaris tribuloides eggs that survived to 48 hours , Kruskal-Wallis Test, p= 0.0215, with standard error bars

and high gas treatments and higher than the high oil + gas treatment [F(6,49)= 13.0933, p<0.0001, Fig 2.12C)].

2.4.3 Larval Viability

Survivorship was significantly higher in experiment 2 compared to experiment 1 (H(1)=5.2854, p=0.0.0215, Fig.2.13). When comparing survivorship within each experiment there was no significance [Experiment 1: (H(3)=1.9830, p=0.5760); Experiment 2: (H(3)=0.6605, p=0.8825)] among treatments or

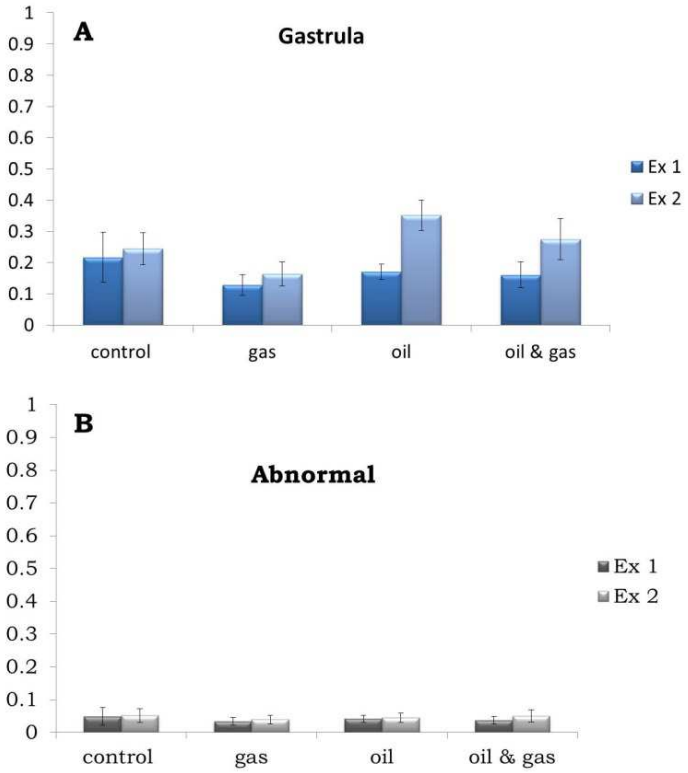


Figure 2.14 The average proportion of *Eucidaris tribuloides* larvae at different developmental stages. Two way anova, p = 0.011

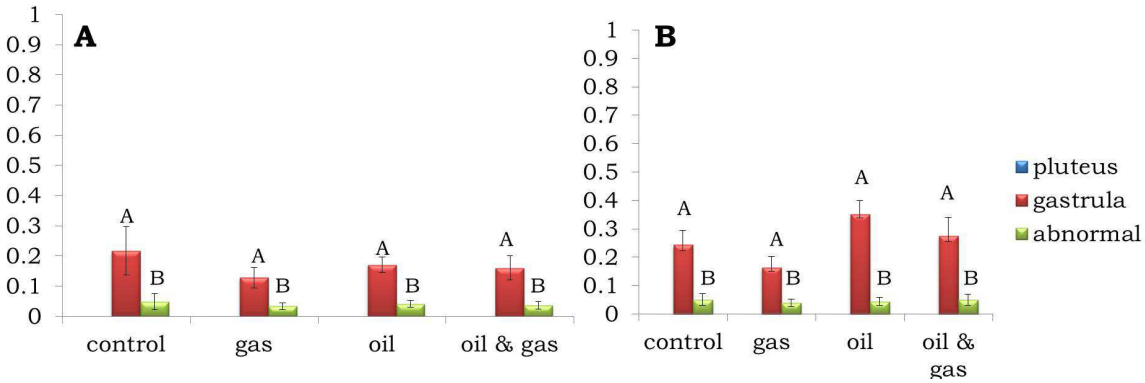


Figure 2.15 The average proportion of *Eucidaris tribuloides* eggs at different developmental stages for experiment 1 p<0.0001 and B) experiment 2 p<0.0001, with standard error bars.

concentrations. Experiment 2 had more embryos in the gastrula stage than experiment one [F(6,49)= 13.0933, p<0.0001, Fig 2.14A)]. No difference was seen in the proportion of abnormal embryos among treatments and experiments (Fig. 2.14B). There were no significant differences among the treatments for gastrula or for abnormal embryos within each experiment. However, there was significantly more gastrula than abnormal in both

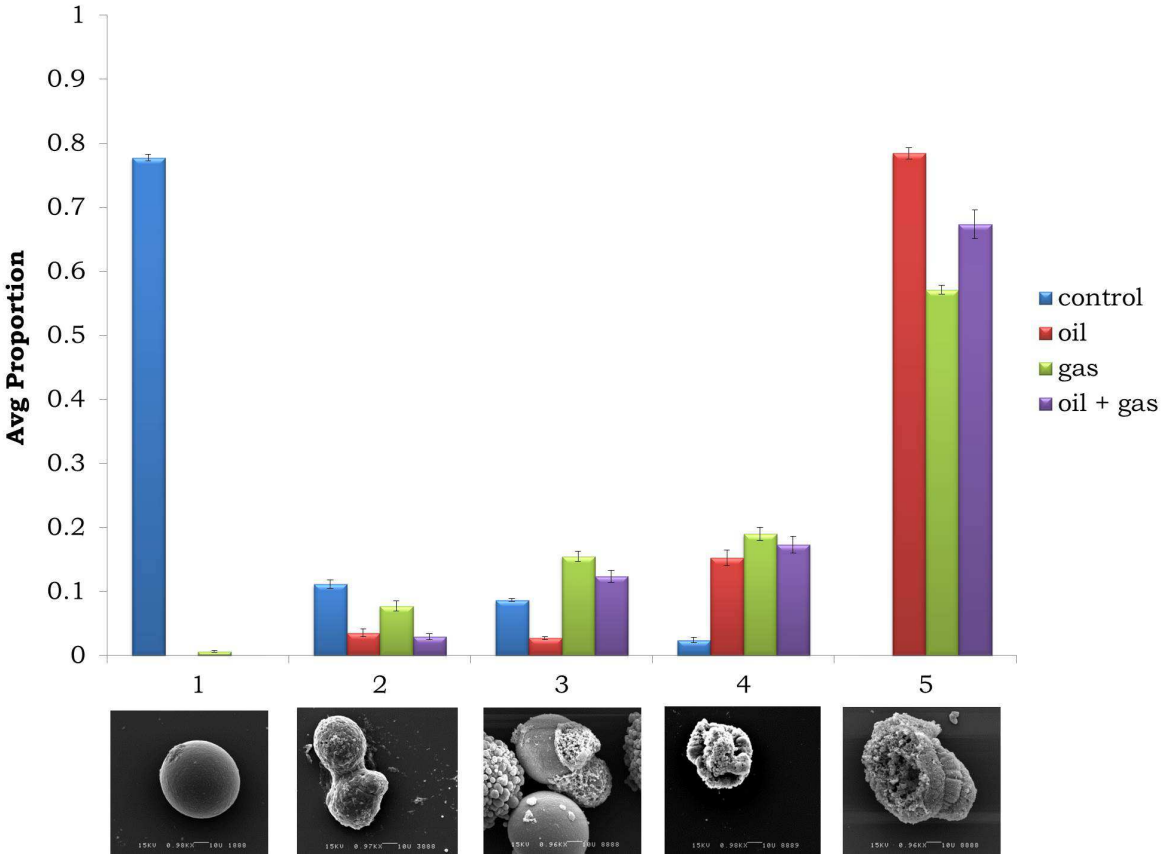


Figure 2.16 *Eucidaris tribuloides* eggs counted and quantified by damage sustained, 1= Normal egg while 5= Severely Abnormal Egg. Statistical significance of p = 0.0028, with standard error bars

experiments [F(8,162)= 12.762, p<0.0001, Fig. 2.15A)] and [F(8,156)= 18.690, p<0.0001, Fig. 2.15B)]. No embryos were seen in the pluteus stage in either experiment.

2.4.4 Scanning Electron microscopy

The controls had the highest proportion of normal, undamaged eggs, while the treatments had the most damaged eggs (H(2)=28.636, p=<0.0001, Fig. 2.16). When categorized based on type of damage sustained, there were significant differences between the oil and the oil + gas treatment: oil [F(5, 112) = 7.408, p<0.0001, Fig. 2.17], oil and gas [F(5, 153)=3.119, p=0.0104, Fig. 2.17] and no significance between the gas treatments (ANOVA, p=0.131, Fig. 2.17). The controls had the highest proportion of eggs in category 1 (normal). The gas treatment had a significantly higher proportion of shrunken eggs (category 5). Oil and oil + gas had more hole and sieving eggs (category 6).

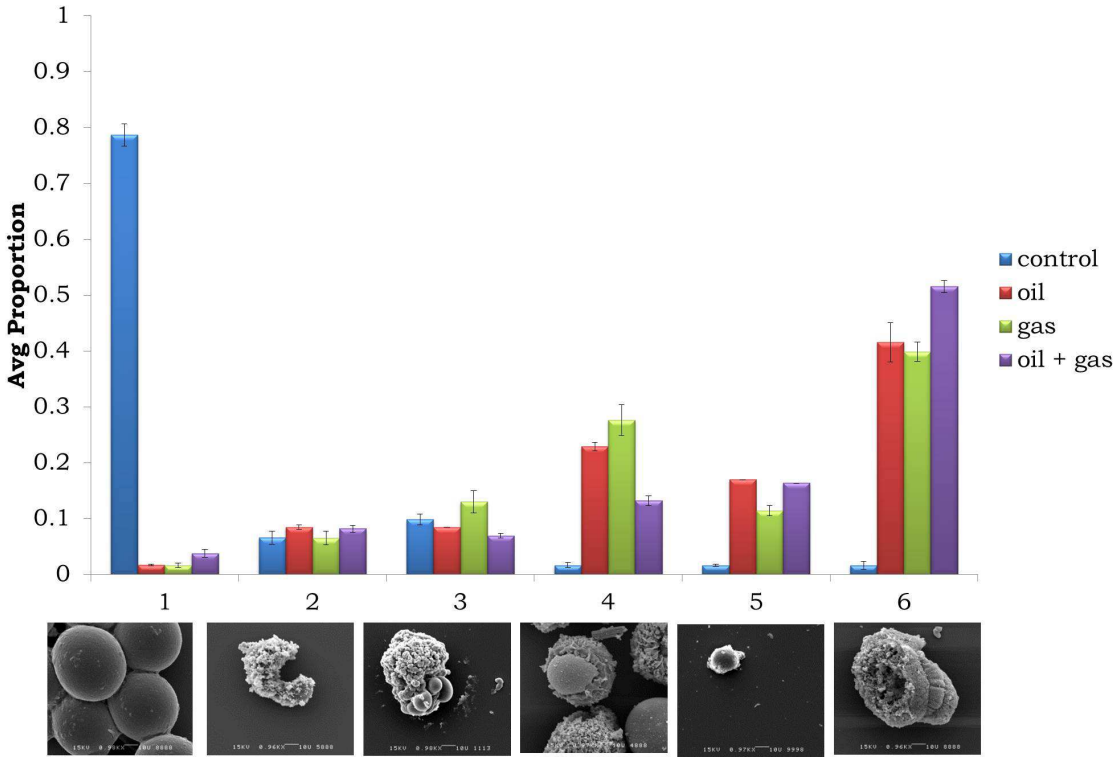


Figure 2.17 *Eucidaris tribuloides* eggs categorized by damage sustained, 1= smooth/none, 2=misshaped, 3=holey, 4= sieving layers, 5= shrunken, 6= holey & sieving. Statistical significance by Tukey's pair wise test of p=0.0104 (oil + gas) and p<0.0001(oil), with error bars with standard error.

2.5 Species Comparison

In experiment 1, *L. variegatus* had a significantly greater survivorship than *E. tribuloides* for all treatments [Gas (H(1)=7.0877, p=0.0078), Oil (H(1)=7.8832, p=0.0050), Oil + Gas (H(1)=8.2256, p=0.0041)Fig 2.18A] except the control (Kruskal-Wallis, p=0.1066). In the second egg dosing experiment, oil was the only significant treatment among species (H (1)=10.0571, p=0.0015, Fig 2.18B) [Kruskal-Wallis, Control, p=0.5623; Gas, p=0.7586; Oil + Gas, p=0.7825].

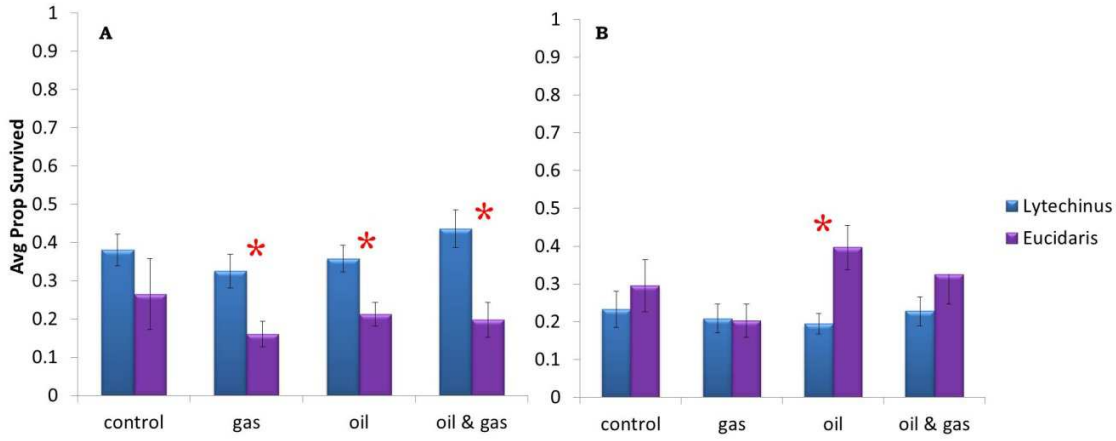


Figure 2.18 Comparison of average proportion survived by treatment between the two urchin species in experiment 1(A) and 2 (B). Nonparametric Kruskal-Wallis test, * above treatment signifies significant $p < 0.05$

2.6 Discussion

Overall, oil, gas and the combination of the two had a negative effect on egg quality, fertilization, and larvae development and survival. Yet, higher pollutant concentrations did not always translate to decreased fertilization and survival. Likewise, lower fertilization did not necessarily mean reduced larval viability. What is perhaps most striking about this study is despite the severe damage the eggs endured from the petroleum treatment, fertilization was successful and larvae were produced.

Sperm concentrations only seemed to influence fertilization success in the oil treatment for *L. variegatus*. A polynomial trendline for the control indicates lower fertilization at the lowest and highest sperm concentrations. The latter is likely a result of polyspermy, where more than one sperm fertilizes an egg leading to lack of successful fertilization or developmental failure (Levitan *et al.* 2007). It seems that even small amounts of oil influences fertilization. When sperm densities are low, small amounts of

oil impede fertilization success, but when sperm densities are high, the oil appears to help prevent polyspermy. This is likely due to the viscous layer that makes sperm penetration slightly more challenging and therefore decreasing polyspermy.

Fertilization varied by species and by experiment. For *L. variegatus*, oil + gas treatment decreased fertilization compared to controls in both experiments, but gas reduced fertilization in eggs only after being dosed for two additional hours (experiment 2). In *E. tribuloides*, only gas in experiment 1 significantly reduced fertilization. This is unexpected since eggs soaked in gasoline for two hours did not have a significant effect on fertilization. SEM images demonstrated that gasoline tended to shrink eggs. It could be that the WAF did not allow for homogeneous mixing of the gasoline or that after two hours some of the gasoline evaporated making it less toxic. The combined effect of gasoline and oil decreased fertilization in *L. variegatus*.

Larval viability differed between experiments and species. *Lytechinus variegatus* demonstrated reduced larval viability after being soaking in pollutants for an additional two hours. *Eucidaris tribuloides* showed the oppose trend. The first experiment viability was higher across all treatments even controls. It is possible that in this species, eggs have reduced viability as they age, thus reducing fertilization in the presence and absence of pollutants. *Lytechinus variegatus* demonstrated an odd pattern where fertilization at the high concentrations of pollutants was significantly lower than the low concentrations but not the controls. There is no clear explanation as to why this might occur; it is possible that polyspermy is reducing fertilization in the controls, but the low concentration of the treatment kill some of the sperm reducing the risk of polyspermy. *Eucidaris tribuloides* had higher larval viability in the second experiment. Because sperm has reduced viability after 2 hours (Oliver and Babcock 1992), different males were used in the second experiment. The sperm from these males may be more compatible with these females. It is also possible that after the eggs age, they become less choosy and fertilization becomes easier (Levitan 1993). *Lytechinus variegatus* had a higher survival rate across all the treatments compared to *E. tribuloides*. The developmental stages differed between the two species as well, as *L. variegatus* seems to have a faster developmental rate. *L. variegatus* were in multiple stages of development,

including pluteus and gastrula, whereas *E. tribuloides* only reached the gastrula stage in the same time period. Overall the number of abnormal larvae was low.

The SEM images revealed extensive degradation and altered egg morphology. In the *L. variegatus* eggs, the highest categorical damage was with the sieving layers, while the highest magnitude among the treatments was the oil and gas, ranking 5. Some treatments had significant effects on the egg, gasoline, for example, tended to cause shrinkage of the egg. This shrinkage could be caused by osmosis, with the gasoline dehydrating the egg. The *E. tribuloides* eggs were smaller overall than the *L. variegatus* eggs, but they followed similar patterns. The highest categorical damage was holey and sieving layers in the oil and gas treatment and the highest magnitude was rank 5 in the oil treatment.

2.7 Conclusion

Massive petroleum events have been shown to be detrimental to the surrounding ecosystem. Here we demonstrated the adverse effects of petroleum pollutants on echinoid reproduction and larval development. Different species of echinoids possess unique tolerance thresholds when exposed to petroleum pollution. The overall effects of this study were decreased fertilization and survivorship in both test species, as well as noticeable degradation of the egg layers. Although fertilization and survivorship occurred, low levels of petroleum pollutants had detrimental effects on the morphology of the eggs (Figs 2.8, 2.9, 2.16, and 2.17).

Echinoids are merely a small percent of broadcast spawners inhabiting a marine ecosystems; many other species including corals, crustaceans, cephalopods like squid, and multiple species of fish also reproduce by spawning events at various times throughout the year. As these events are imperative to the survival and future growth of a species, any damage during this time can be detrimental. Additional studies, specifically examining the egg layers, are needed to further our understanding of how fertilization is possible after severe egg degradation. Studies tracking the long term growth of exposed eggs could also be used to observe the possibility of developing to adulthood and the potential for those adults to then spawn and produce viable gametes. This would allow for examination of any transgenerational or latent effects of the initial petroleum pollution to future generations of echinoid species.

Chapter 3

3.1 Discussion

Massive oil spills like the Deep Water Horizon and the Exxon Valdez receive high priority because of the magnitude and national exposure; yet, anthropogenic pollutants introduced daily are not widely studied and may reduce marine populations significantly (McCook 1999, Nystrom *et al.* 2000, Bellwood *et al.* 2004). Of the 3.5 million tons of oil that is released into the ocean every year, most of which are from the daily input of bilge water (Wiggins 2000). Since there is no consistency on the quantity of petroleum pollutants released at a single point (Johnson 2008), most organisms in the water column have no natural defenses against petroleum pollutants (Kvenvolden 2003).

Since the concentration of oil and gas in bilge water varies by vessel, researchers use WAFs as a way to standardize the concentrations assessed during an experiment (Singer 2000). Many researchers have used WAF's to examine the effects of pollutants on fertilization and development (Bellas *et al.* 2013, Nichol *et al.* 1977, Berdugo *et al.* 1977, Fadlallah 1983, and Negri & Heywood 2000). Experimental concentrations were chosen based on the EPA's limit of allowable gas and oil and then expanded to show a wide range of possible concentrations.

The species chosen for this experiment were selected based on geography and availability at the time of experiment. The two species used were physiologically different from each other. *Eucidaris tribuloides* is a primitive sea urchin that closely resembles its 200 million year old ancestors, whereas *Lytechinus variegatus* is a more recent lineage (Keir 1974, Smith 1984). It is possible that a species-specific response to oil pollutants could occur.

The goals of this project were to answer four specific questions to understand the effects of petroleum pollutants, specifically bilge water, on fertilization success and larval viability:

- Will the integrity of the outer egg layers be compromised by oil and/or gas?
- If the integrity of the outer egg layer is compromised by pollutants, will fertilization be affected?

- Will fertilization success vary among oil and/or gas concentrations?
- Will petroleum pollutants affect embryo viability after 48 hours?

3.1.2 Egg integrity

Echinoids are frequently used for fertilization, physiology, and molecular biology research; yet few studies focus on fertilization from the perspective of the egg (Wessel *et al.* 2004). Sea urchin eggs are roughly 100 microns in diameter, 50 ng in total mass, and are all at the same developmental stage when extruded (Wessel & Vazquier 2004). Because of this uniformity, healthy eggs should appear similar. The primary goal of this study was to use the SEM to examine the sea urchin eggs for degradation..

Analysis of the SEM images revealed egg degradation across all concentrations and all treatments, with the highest degradation in the highest concentration, i.e., 1000ppm. The magnitude of the damage correlates with the categorical damage sustained to the eggs. In the *L. variegatus* eggs, the highest categorical damage was with the sieving layers, while the highest magnitude among the treatments was the oil + gas treatment, ranking 5. Some treatments had significant detrimental effects on the egg. Gasoline, for example, tended to cause shrinkage of the egg. This shrinkage could be caused by osmosis, with the gasoline dehydrating the egg. The *E. tribuloides* eggs were smaller overall than the *L. variegatus* eggs, but they followed similar degradation patterns. The highest categorical damage was holey & sieving layers in the oil + gas treatment, and the highest magnitude was rank 5 in the oil treatment.

3.1.3 Fertilization

I conducted two fertilization experiments: one where both gametes were introduced to the pollutants simultaneously and the second experiment where the eggs were dosed for two hours prior to introducing sperm to the petroleum products. One of the challenges of this project was finding sufficient number of sea urchins and males in particular. Sea urchins are not sexually dimorphic; therefore, males and females cannot be distinguished from each other. Consequently, sea urchins must be injected with potassium chloride (0.55M) and extrude gametes to distinguish gender. Previous studies state that sperm can be freeze-dried and reactivated hours later (Foltz *et al.* 2004). This

did not prove to be as the case for *L. variegatus* sperm, which were not reactivate and thus fertilization did not occur. Since I was unable to freeze and reuse the sperm from the first experiment, new males were induced to spawn for the second experiment. With many new graduate students helping with counting the fertilization envelopes and counting the eggs to be used in the viability experiment, there were some errors reported, such as more larvae in viability experiment than fertilized eggs counted in the fertilization experiment. If an inconsistency was found the data from that set was not used in the statistical calculations. This accounts for inconsistencies in the sample sizes in the low and medium concentrations.

There was higher fertilization success in experiment 1 in *E. tribuloides*, but not in *L. variegatus*. The gas concentrations reduce the amount of fertilization, the control averaged at about 80% fertilized while the high gas concentration at about 30% fertilized. This decrease may be due to the smaller egg size in *E. tribuloides.* Research has shown that small egg size makes it more difficult for sea urchin sperm to find; therefore, decreasing fertilization or leading to other adaptations such as long lived sperm (Levitan 1993 and 2000). If gas further diminishes the egg size, then fertilization will be further reduced. In the egg dose experiment, small egg size was seen in the gas treatments, while oil treatment alone was not affected.

The reduced fertilization in *L. variegatus* oil treatments could be attributed to the sperm concentrations. A polynomial trendline for the control indicates lower fertilization at the lowest and highest sperm concentrations. The latter is likely a result of polyspermy, where more than one sperm fertilizes an egg leading to lack of successful fertilization or developmental failure (Levitan *et al.* 2007). It seems that even small amounts of oil influences fertilization when taking into account sperm concentration. When sperm densities are low, small amounts of oil impede fertilization success, but when sperm densities are high, the oil appears to help prevent polyspermy. This is likely due to the viscous layer that makes sperm penetration slightly more challenging and therefore decreasing polyspermy. Either polyspermy does not occur in *E. tribuloides* or experimental sperm concentrations were not high enough to induce polyspermic fertilization.

3.1.4 Viability

In the viability experiments, eggs (fertilized and unfertilized) were counted and placed in mason jars with penicillin and streptomycin antibiotics and left undisturbed for 48 hours. This is the timeframe for larvae before they would need to be fed, and they should undergo metamorphism to the pluteus stage under normal culturing conditions (Foltz *et al.* 2004). *Lytechinus variegatus* should reach the gastrula stage at 11 hours and the pluteus stage at 20 hours (Foltz *et al.* 2004). *Eucidaris tribuloides* is a "primitive" sea urchin and the development in this species is slower (Bennett 2012). The gastrula stage begins at 18 hours and continues to 21 hours, while the pluteus stage begins at 44 hours and continues to 75 hours (Schroeder 1981).

As with the fertilization counts, many graduate students helped with the counting, and any discrepancies during the first few trials were examined and not used if inconsistent with the number of fertilized embryos placed in the culture. In experiment 1, *L. variegatus* had a higher survival rate (40-50% survivorship) across all treatments compared to rates of *E. tribuloides* (15-27% survivorship). In experiment 2, *L. variegatus* has a lower survival rate (19-24% survivorship) across the treatments compared to the rates of *E. tribuloides* (20-40% survivorship).

The difference in survivorship between the *L. variegatus* experiments can be explained by polyspermy and the egg degradation. While fertilization success was higher, viability was reduced in experiment 2 (19-24%), compared to experiment 1 (40-50%). While the eggs in experiment 1 were only exposed to the pollutants for a short period (<5 mins before fertilization), the eggs from experiment 2 were exposed for 2 hours before fertilization could occur. Significant damage occurred to the eggs during this time. The jelly coat and the vitelline layer are the main block for sperm. The eggs at the time of fertilization release protease activity extracellularly, which is a polyspermy block that hardens the vitelline layer of the egg, stopping any sperm from penetrating the cell further (Vacquier 1973). With the damage to these outer layers, the protease would not be able to harden the layer and stop penetrations to the egg. This can cause multiple problems including lower survivorship and developmental problems.

From the SEM images, the eggs were badly damaged in most treatment concentrations (Figs 2.8, 2.9, 2.16, and 2.17). The higher the concentration the higher the

damage, but the low concentrations still had damage as well (including holes, sieving and shrinkage). SEM images demonstrated that gasoline tended to shrink eggs. It could be that the WAF did not allow for homogeneous mixing of the gasoline or that after two hours some of the gasoline evaporated making it less toxic. Those eggs that were not completely destroyed may have fertilized (i.e., the fertilization envelope was raised) but the abnormalities from the damage sustained could arrest further development. *Lytechinus variegatus* had multiple stages of development, including pluteus and gastrula, whereas *E. tribuloides* reached the gastrula stage in the same timeframe. Although pollutants could have slowed down development, the controls were at the same developmental stage as the treatments, therefore eliminating this hypothesis. There were a minimal number of abnormal larvae, which was surprising. Kobayashi (1977) studied the effects of oil and dispersants on echinoid embryos and developmental stages of several echinoids. He found that the pollutants caused the pluteus stage contained more abnormal larvae than the earlier stages of development.

3.2 Conclusion

Massive petroleum spills have shown to devastate an ecosystem. Here we demonstrated the adverse effects of low concentrations of petroleum pollutants on echinoid reproduction and larval development. Different species of echinoids possess unique tolerance thresholds when exposed to petroleum pollution. The overall effects of this study were decreased fertilization and survivorship in both test species, as well as noticeable degradation of the egg layers. Although fertilization and survivorship occurred, low levels of petroleum pollutants had detrimental effects on the morphology of the eggs.

Broadcast spawning is a common reproductive strategy. Any damage to the gametes during reproduction can be detrimental to the species' population. With many fisheries and marine ecosystems in jeopardy, successful fertilization and development is important for effective management of these fisheries. Frequent surveys of pollutant levels in areas frequented by vessels would be useful to mitigate the input of petroleum products via bilge discharge. Additional studies, specifically examining the egg layers, are needed to further our understanding of how fertilization is possible after severe egg

degradation and if development will continue to reproductive maturity. This would allow for examination of any transgenerational or latent effects of the initial petroleum pollution to future generations of broadcast spawners.

Many of the most diverse marine communities are in shallow waters and are vulnerable to coastal anthropogenic pollutants. In an effort to minimize the detrimental effects of anthropogenic pollutants on the marine environment, the EPA restricts the amount of petroleum pollutants allowed to be discharged in our waters. We have tested the allowable limit set forth by the EPA, and the effects to the egg and the egg layers is damaging and fertilization success and larvae viability were reduced. Based on these data, the existing standards on the amount of petroleum that is allowed to be discharged should be revisited.

Appendix: Supplemental Material

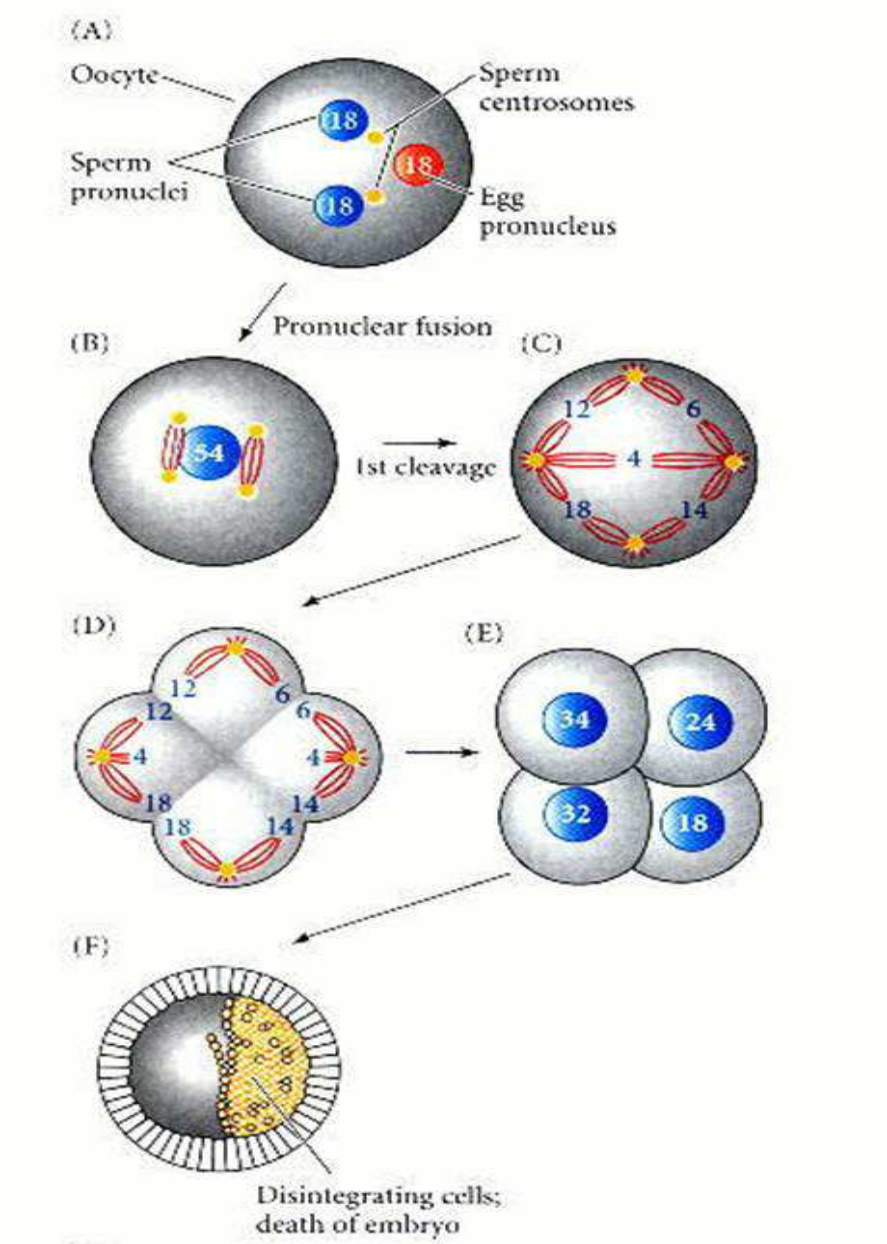


Figure A.1 Dispermic egg with Aberrant development. A. Combination of the three haloid nuclei, with 18 chromosomes. B. Formation of mitotic poles and division of the two sperm centrioles. C. Random arrangement of 54 chromosomes on spindles. D. As anaphase begins, the chromosomes are divided and pulled to the corresponding poles if the spindles. (E) With the random assortment of chromosomes, four cells are formed. F. This can causes disintegrations in the cells and early death in the embryos. (Gilbert 2000)

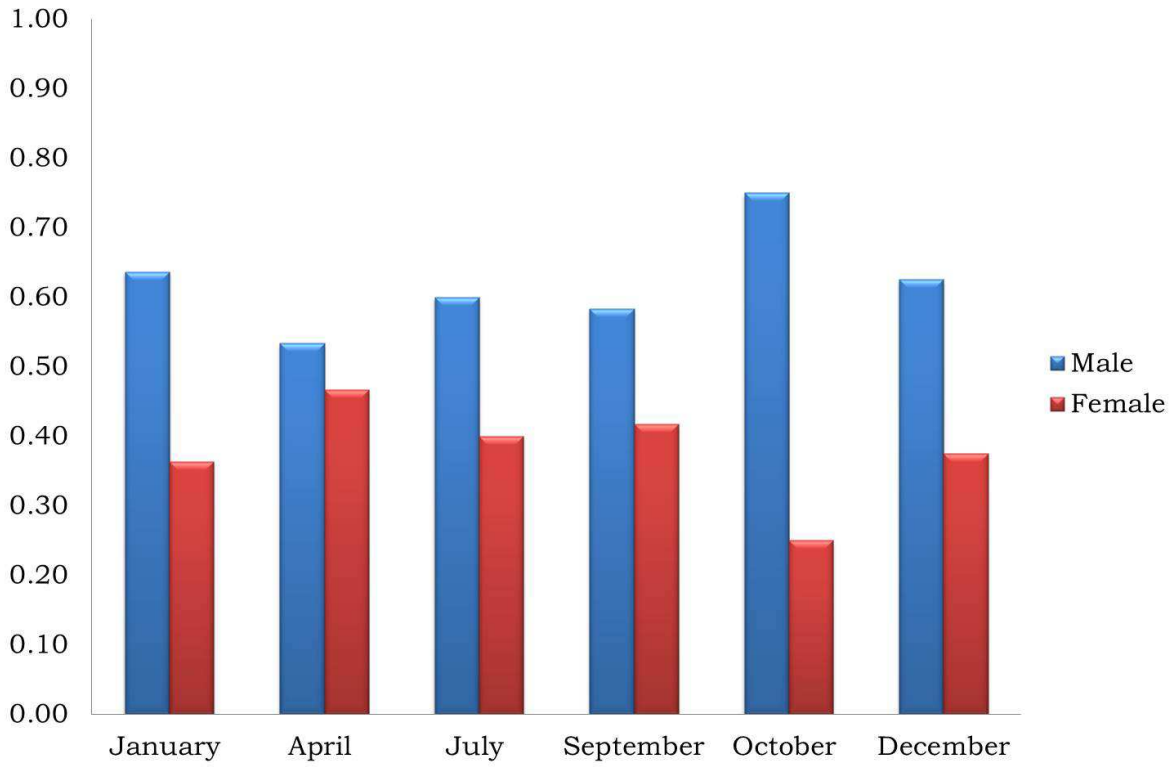


Figure A.2. Sex ratios in *Lytechinus variegatus* per trial

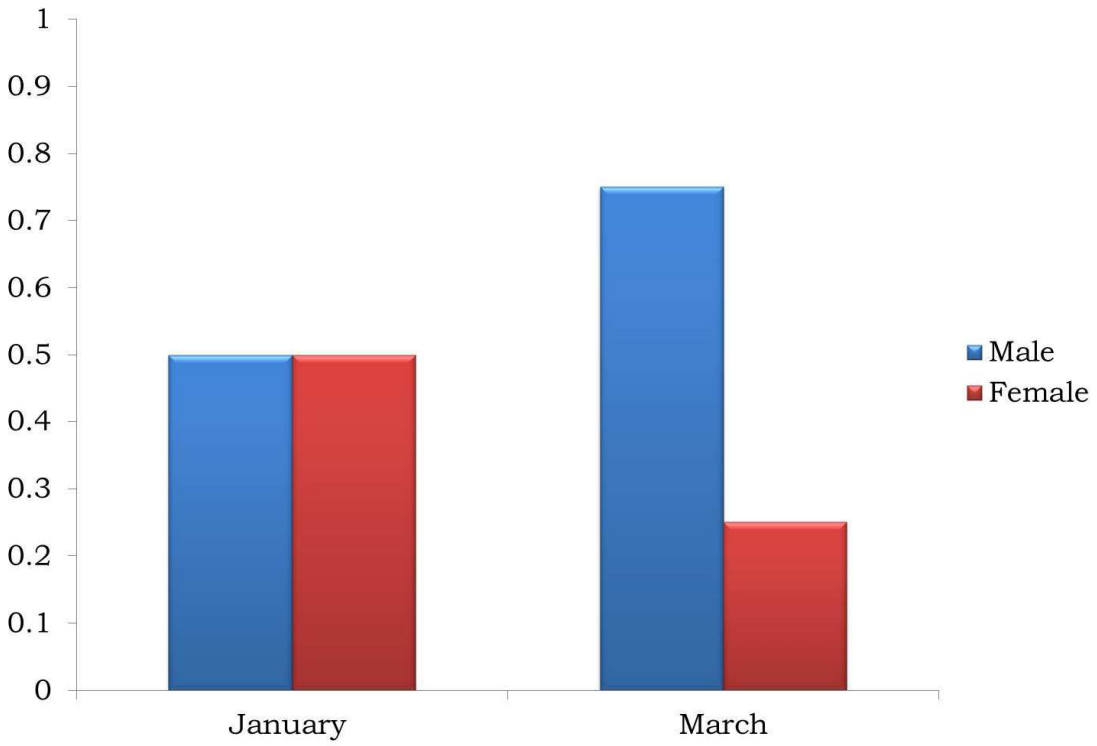


Figure A.3 Sex ratio for *Eucidaris tribuloides* per trial

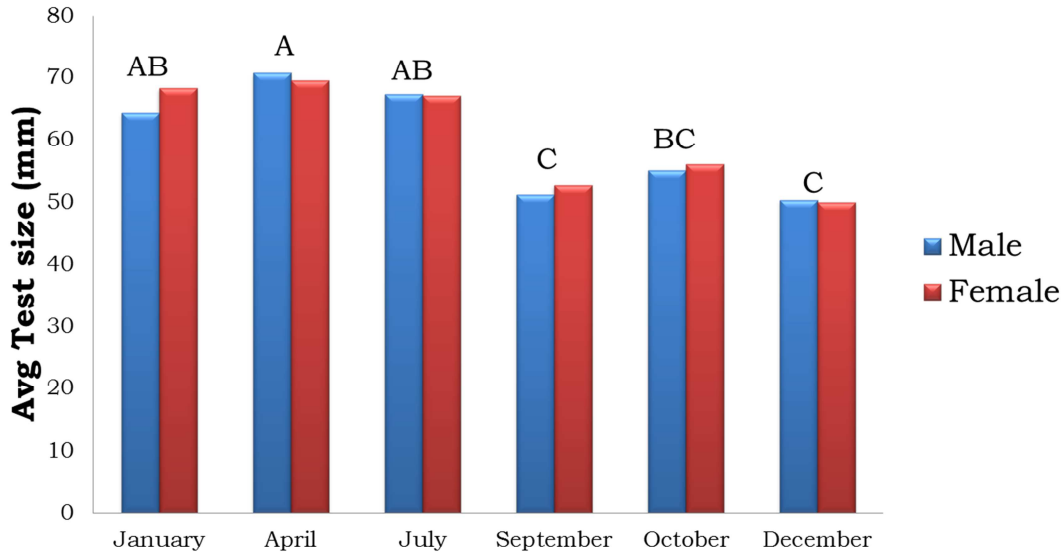


Figure A.4 Average Test Size of *Lytechinus variegatus* by Trial Month. Significance between the months, p<0.0001 not the sex

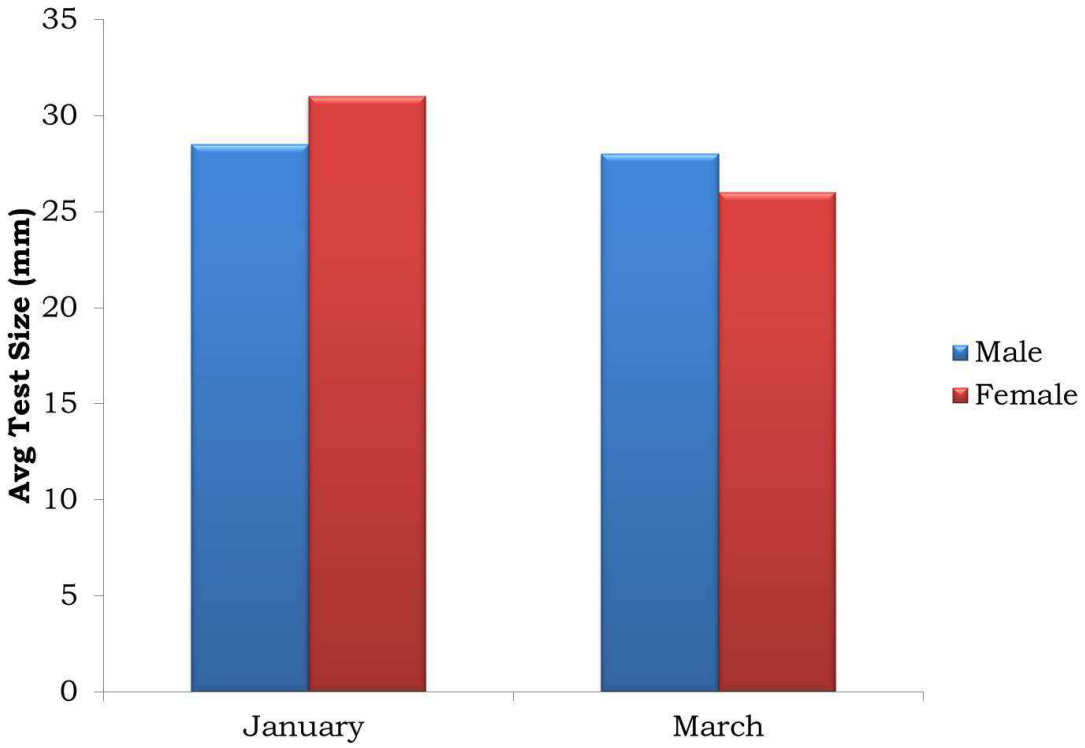


Figure A.5 Average Test Size of *Eucidaris tribuloides* by Trial Month

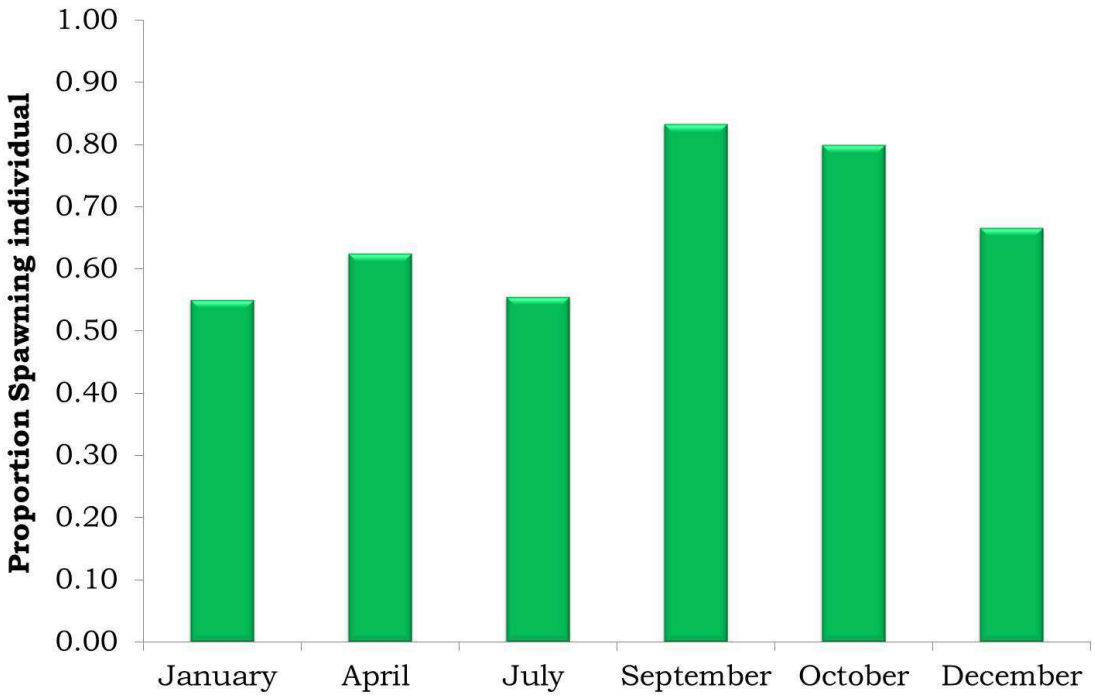


Figure A.6 Proportion of spawning individuals among *Lytechinus variegatus*

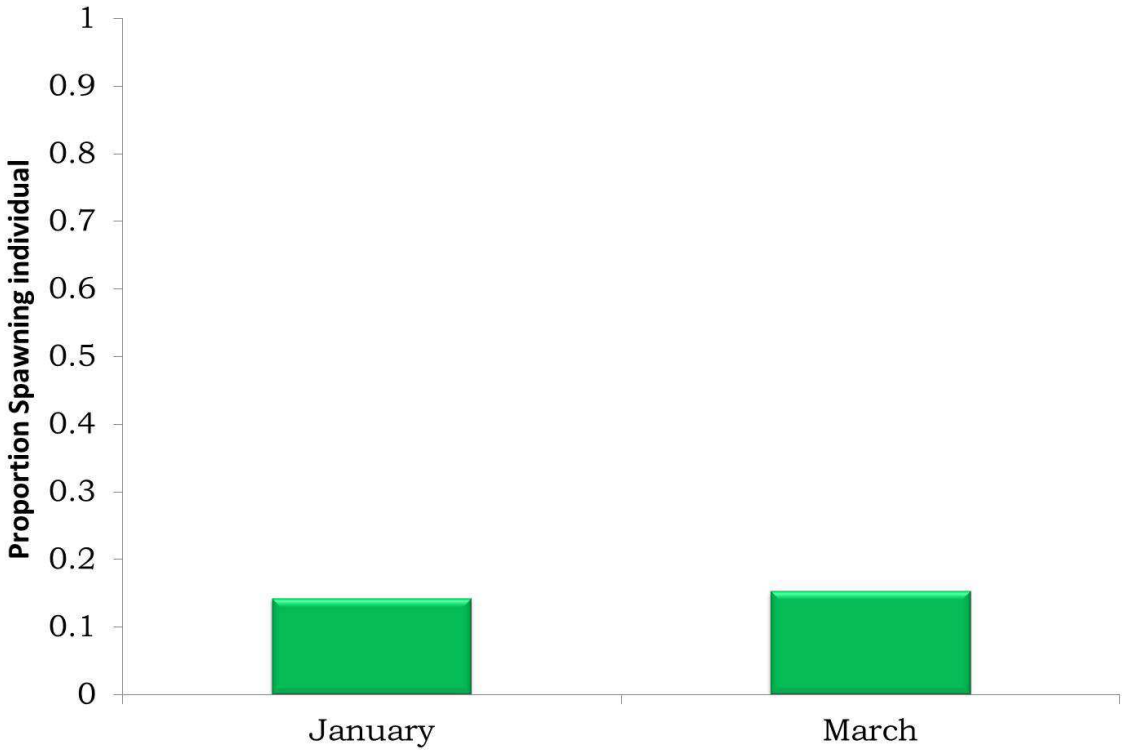


Figure A.7 Proportion of spawning individuals among *Eucidaris tribuloides*

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